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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
This proposal will test a unique hypothesis that Src-family kinases direct the ErbB receptor traffic into an intracellular compartment where proliferation signals are generated. This hypothesis is based on observations that while Cbl proto-oncoprotein facilitates the down-regulation of ErbB receptors from cell surface and functions as a negative regulator, Src tyrosine kinase enhances paradoxically enhance both the internalization of EGFR and the EGFR-mediated mitogenic signals. The proposed studies will test this hypothesis using mammary epithelial cells made to overexpress EGFR or ErbB2 together with Src. The insights gained from this model system will be directly relevant to a large proportion of breast cancers where ErbB receptors and Src-family kinases are co-overexpressed. Validation of our hypothesis will represent a shift in the current paradigm of normal and aberrant ErbB receptor signaling and may provide novel targets for therapeutic intervention relevant to ErbB-overexpressing breast cancers, which carry a significantly worse prognosis and are frequently hormone-unresponsive.

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Introduction:

This application proposes to test a unique hypothesis that Src-family kinases direct the ErbB receptor traffic to recycling endosomal compartment, where proliferation signals are generated from the internalized pool of the activated ErbB receptor tyrosine kinases. This hypothesis is based on previous observations that the potency of mitogenic signals emanating from ErbB receptors correlates directly with the ability of these receptors to avoid the lysosomal pathway of degradation and to be sorted into the recycling pathway instead. For example, ErbB1 (EGFR) is targeted to lysosomal as well as recycling pathways, whereas the more potent ErbB2 receptor is primarily recycled. Work in our laboratory has defined the Cbl proto-oncoprotein as a novel regulator of this process. Cbl enhances the delivery of ErbB1 into the lysosomal pathway. In contrast, Cbl does not interact efficiently with ErbB2, which is primarily recycled. Paradoxically, Src tyrosine kinase enhances the EGFR internalization while at the same time enhancing the EGFR-mediated mitogenic signals. This led us to hypothesize that Src directs ErbB receptors away from lysosomes and into a recycling endosomal compartment where these receptors continue to transmit proliferation signals. The proposed studies are designed to test this hypothesis using mammary epithelial cells made to over-express EGFR or ErbB2 together with Src. This model system will provide insights directly relevant to a large proportion of breast cancers where ErbB receptors and Src-family kinases are co-over-expressed. Validation of our hypothesis will represent a shift in the current paradigm of normal and aberrant ErbB receptor signaling and may provide novel targets for therapeutic intervention relevant to ErbB-over-expressing breast cancers, which carry a significantly worse prognosis and are frequently hormone-unresponsive.

Body of Report:

During the second year, we have undertaken studies proposed under Tasks 3-5 of the modified SOW. A large series of experiments indicated that the pMSCV-blast vector system we devised earlier failed to direct high-level expression of EGFR. Therefore, we redesigned our strategy for co-expression of ErbB2 or EGFR with Src, using a combination of pMSCV-puro based EGFR or ErbB2 constructs together with pMSCV-hygro-Src. As a result, we have now established two distinct series of human mammary epithelial cell lines that over-express EGFR, ErbB2, Src, EGFR plus Src or ErbB2 plus Src. We have also initiated the analyses of these cellular models to test the tenants of our proposed hypothesis.

Our preliminary analyses had suggested that the rate of EGFR degradation may be altered in human mammary epithelial cells immortalized with HPV E6/E7 (in 16A5 cell line), which led us to identify an h-TERT-immortalized mammary epithelial cell line as an alternative(last year's report). More careful analysis demonstrated that 16A5 and 76N-TERT cells show an essentially identical kinetics of EGFR degradation upon EGF stimulation when analyzed biochemically (**Fig. 1**). Based on these results, and the detailed studies reported last year, we selected the following three human mammary epithelial cell lines to generate stable transfectants over-expressing EGFR, ErbB2, Src, EGFR plus Src or ErbB2 plus Src: 16A5 (E6/E7-immortalized) and 76N-TERT (hTERT catalytic subunit immortalized) both derived from 76N cell strain with basal cell characteristics; M2-E6E7, an HPV E6/E7-immortalized derivative of the M2 milk cell strain, representing luminal mammary epithelial cells.

Our earlier studies (reported last year) had demonstrated the suitability of the pMSCV-based retroviral infection approach to direct the over-expression of ErbB2 in human mammary epithelial cells. We therefore extended this approach for the over-expression of ErbB2, EGFR and Src, individually and in combination. We co-transfected the pMSCV-blast vector or pMSCV-blast-EGFR construct together with the packaging vector pIK into the packaging cell line TSA54. The supernatants of these transiently-transfected cells were used to infect 16A5 or 76N-TERT cell lines. Following infection, the cells were selected in blasticidin (2.5 ug/ml, based on prior kill curves) and resistant cell lines were obtained. The levels of EGFR expression on these cell lines were assessed biochemically using anti-EGFR immunoblotting and by fluorescence-activated cell sorter (FACS) analysis. However, even after repeated attempts, we were unable to obtain a significant over-expression of EGFR even though the cells were blasticidin-resistant.

To circumvent this problem, we cloned the EGFR cDNA in the pMSCV-puro vector. Side-by-side comparison in transiently transfected 293T cells revealed that the pMSCV-blast-EGFR construct directed an extremely low level of EGFR expression compared to that using the pMSCV-puro-EGFR construct ; a similar difference was observed for another gene (human sprouty 2) cloned into the pMSCV-puro and pMSCV-blast vectors (data not shown). The reason for the inefficiency of the pMSCV-blast-EGFR vector to support expression remains unclear. To avoid further problems, we switched to the pMSCV-puro-EGFR vector for the over-expression of EGFR in human mammary epithelial cells.

The pMSCV-puro, pMSCV-puro-EGFR or pMSCV-ErbB2 constructs were transiently transfected into the packaging cell line and the retroviral supernatants were used to infect 16A5, 76N-TERT and M2-E6E7 mammary epithelial cell lines. Stable puromycin-resistant cell lines were obtained and the over-expression of EGFR or ErbB2 on these singly transfected cells was verified by FACS analysis and immunoblotting of cell lysates (**Fig. 2-8**). In the next step, we generated retroviral supernatants using pMSCV-hygro or pMSCV-hygro-Src constructs. The supernatants were used to infect the EGFR or ErbB2 over-expressing cell lines. FACS and immunoblotting analyses were again performed to ensure the continued over-expression of EGFR or ErbB2 on these cells (**Fig. 2-8**). Immunoblotting analysis with a monoclonal anti-Src antibody (provided by Dr. Sheila Thomas, Beth Israel-Deaconess Medical Center, Boston, MA) demonstrated the over-expression of Src in doubly-transfected cells as well as in cells transfected with Src alone (**Fig. 2-8**).

Table I provides a summary of the various transfected cell lines that have been obtained. The expression analyses have been carried out on the 76N-TERT and 16A5 transfectants. All single as well as the double transfectants, except for 16A5-EGFR+Src transfectants, were found to over-express the transfected proteins. The 16A5-EGFR+Src transfectant is being re-derived. Analysis of the transfected protein expression in M2-E6E7 transfectants is underway.

Next, we examined the impact of EGFR or ErbB2 over-expression, with and without Src, on the growth of transfected mammary epithelial cell lines. Anchorage-independent growth, a reflection of oncogenic transformation of mammary epithelial cells (Ref #1), was assessed by the ability of cells to form colonies when plated in soft agar. As expected (Ref # 1), the

untransfected and vector-transfected 16A5 cell lines did not form colonies under these conditions. Over-expression of EGFR or ErbB2 led to colony formation in soft agar; this was further increased when Src was also over-expressed (**Fig. 9-10**). Thus, over-expression of EGFR or ErbB2 enhances the anchorage-independent growth of mammary epithelial cell line 16A5 and this trait is further increased by Src.

Given the important role of ErbB receptors and Src in regulating cell differentiation, migration and extracellular matrix signaling, we have also begun to characterize the ability of transfected mammary epithelial cells to form organized structures when grown on matrigel; untransformed mammary epithelial cells are known to form well polarized acini-like spheroids under these conditions, whereas transformed cells make abnormal structures (Ref # 2 and 3). Untransfected, vector-transfected and Src-over-expressing 76N-TERT cells formed small spheroids (**Fig. 11**), apparently composed of a single cell layer (data not shown). In contrast, the transformed MDA-MB231 cells grow as large branched structures (**Fig. 11**). Notably, the cells transfected with ErbB2 or ErbB2 plus Src produced more and larger spheroids (**Fig. 11**). Thus, ErbB2 over-expression appears to alter the ability of mammary epithelial cells either to organize into three-dimensional acinar structures or to grow under three-dimensional culture conditions. These studies are now being expanded.

Finally, we have recently established the conditions for subcellular immunolocalization of EGFR and other endocytic proteins in CHO and mouse embryonic cell lines (**see attached manuscript in preparation**). We have now applied these protocols to human mammary epithelial cell transfectants, and expected localization of EGFR, ErbB2 and Src has been established. For example, EGFR and ErbB2 show predominant surface localization on unstimulated cells, and a differential downregulation of these receptors from the cell surface is observed upon EGF stimulation (EGFR is internalized whereas ErbB2 continues to be mainly on the cell surface). Src shows predominant perinuclear localization (**Fig. 12**). Thus, the cell lines as well as the methodology for immunolocalization studies have been established.

Key Research Accomplishments:

- Generated three distinct sets of mammary epithelial cells (16A5, 76N-TERT and M2-E6E7) with over-expression of EGFR, ErbB2, Src, EGFR+Src or ErbB2+Src.
- Established the relative over-expression of EGFR, ErbB2 and Src in transfected cells.
- Provided initial evidence that over-expression of EGFR plus Src or ErbB2 plus Src enhances the growth of human mammary epithelial cells.
- Provided initial evidence that ErbB2 over-expression alters the ability of human mammary epithelial cells to grow in three-dimensional cultures.
- Established EGFR-expressing wildtype and Cbl-deficient MEFs.

- Localized the endocytic step of Cbl-mediated EGFR downregulation to sorting as opposed to internalization.

Reportable Outcomes:

Reagents:

- Generated three distinct sets of mammary epithelial cells (16A5, 76N-TERT and M2-E6E7) with over-expression of EGFR, ErbB2, Src, EGFR+Src or ErbB2+Src.

- Generated a matching pair of HPVE6 and h-TERT immortalized derivatives of an additional human mammary epithelial cell strain 81N.

- Established EGFR-expressing wildtype and Cbl-deficient MEFs.

Publications:

- Duan L. et al. Cbl-mediated ubiquitinination is a sorting signal for degradation of EGFR. Manuscript in preparation (see attached).

Conclusions:

In conclusion, we have established three sets of human mammary epithelial cell transfectants, representing both basal and luminal cells, which over-express EGFR, ErbB2, Src, EGFR plus Src or ErbB2 plus Src. In addition, the appropriate vector control cell lines have also been generated. Initial studies have been performed and these indicate a significant impact of EGFR, ErbB2 and Src on the growth characteristics of transfected mammary epithelial cells. The various assays have been standardized for completion of proposed studies to test the hypothesis that Src regulates the intracellular traffic of ErbB receptor to a recycling endosomal signaling compartment in mammary epithelial cells.

References:

1. Ratsch SB, Gao Q, Srinivasan S, Wazer DE, Band V. Multiple genetic changes are required for efficient immortalization of different subtypes of normal human mammary epithelial cells. Radiation Research, 2001; 155:143-150.

2. Hansen RK, Bissell MJ. Tissue architecture and breast cancer: the role of extracellular matrix and steroid hormones. Endocrine Related Cancer 2000;7:95-113.

3. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat. Cell Biol. 2001;3:785-92.

Fig. 1. Time course of the ligand-induced EGFR down-regulation in mammary epithelial cell lines derived with different immortalizing agents. The indicated cell lines were starved in EGF-free D3 medium for 48 hours prior to stimulation with EFF (100 ng/ml) for the indicated time points. Equal aliquots of lysate proteins were subjected to anti-EGFR immunoblotting (antibody sc003; Santa Cruz Biotech.). 16A5, 16E6P and 76N-Tert are derivatives of 76N normal mammary epithelial cell strain immortalized with HPV16 E6/E7, E6 or h-TERT, respectively. 81E6 and 81N-Tert are E6- and hTERT-immortalized derivatives of normal mammary epithelial cell strain 81N. The kinetics of the loss of EGFR signal is comparable among different cell lines.

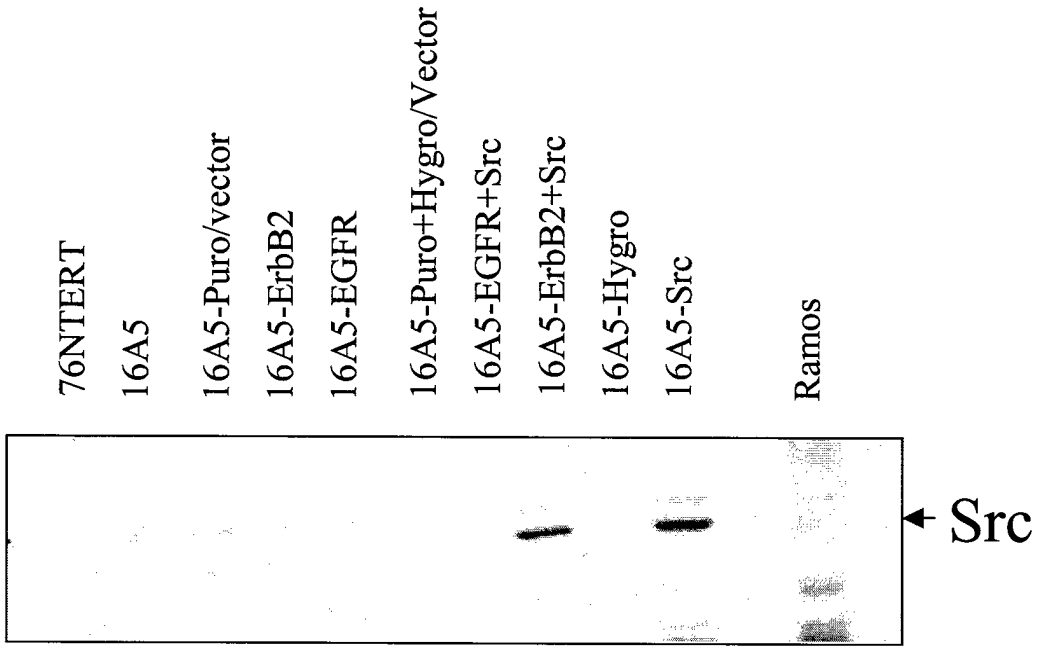


Fig. 2. Immunoblot analysis of Src overexpression in 16A5 transfectants. Cells were starved for 48 hrs by growth in EGF-deficient D3 medium and cell lysates were prepared in RIPA lysis buffer. 50μg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with an anti-Src monoclonal antibody (327). Ramos is a B-lymphoma line. A relatively low exposure is shown to highlight the overexpression of Src; endogenous Src signals were present in all cell lines upon longer exposure.

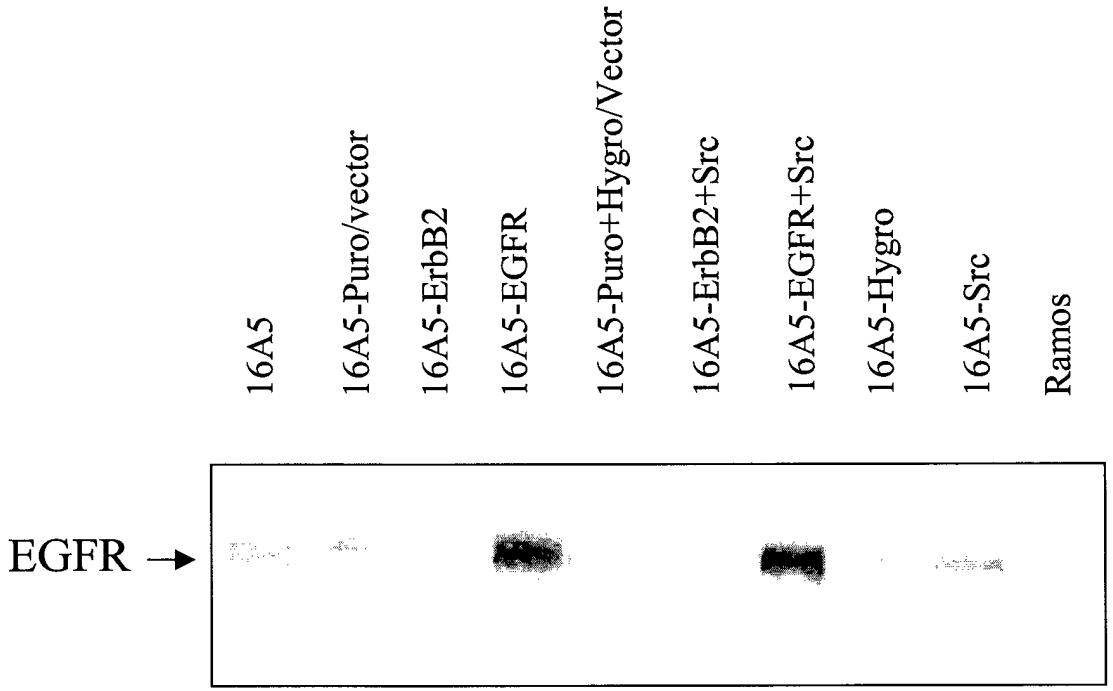


Fig. 3. Immunoblot analysis of EGFR overexpression in 16A5 transfectants. Cells were starved for 48 hrs by growth in EGF-deficient D3 medium and cell lysates were prepared in RIPA lysis buffer. 50µg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with with a cocktail of anti-EGFR monoclonal antibody (NeoMarkers). Ramos is a B-lymphoma line. A relatively low exposure is shown to highlight the overexpression of EGFR; endogenous EGFR signals were present in all cell lines, except Ramos, upon longer exposure.

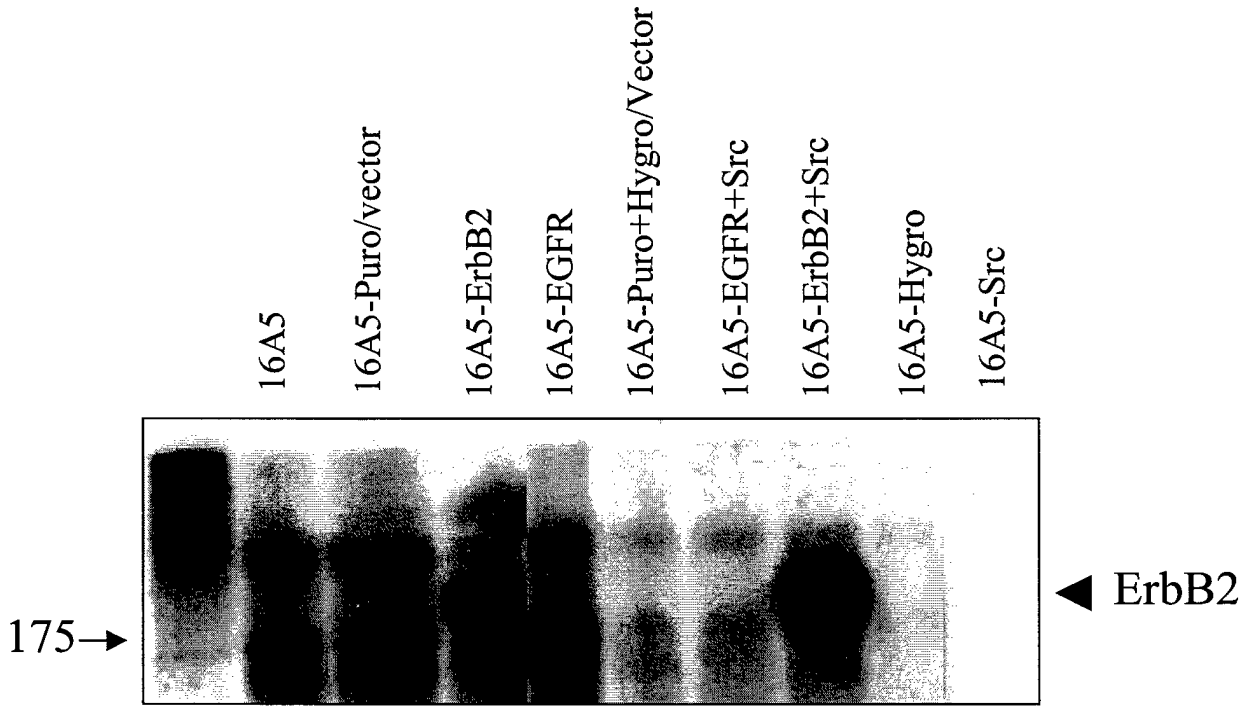


Fig. 4. Immunoblot analysis of EGFR overexpression in 16A5 transfectants. Cells were starved for 48 hrs by growth in EGF-deficient D3 medium and cell lysates were prepared in RIPA lysis buffer. 50µg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with with anti-ErbB2 monoclonal antibody (NeoMarkers). A relatively low exposure is shown to highlight the overexpression of ErbB2; endogenous ErbB2 signals were present in all cell lines upon longer exposure.

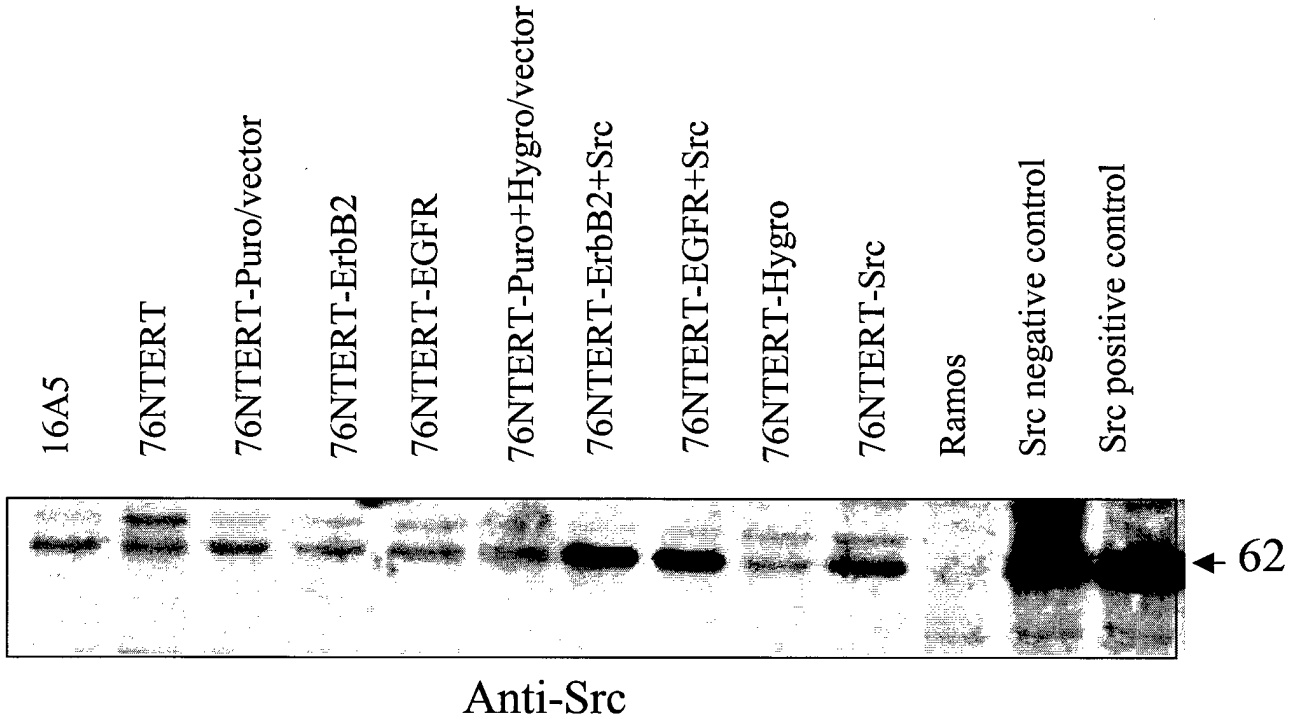


Fig. 5. Immunoblot analysis of Src overexpression in 76N-TERT transfectants. Cells were starved for 48 hrs by growth in EGF-deficient D3 medium and cell lysates were prepared in RIPA lysis buffer. 50μg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with an anti-Src monoclonal antibody (327). Ramos is a B-lymphoma line. Src-nagative and Src-positive controls are Src-/- and Src+/+ mouse embryonic fibroblasts. A relatively low exposure is shown to highlight the overexpression of Src; endogenous Src signals were present in all cell lines, except Src-negative control, upon longer exposure.

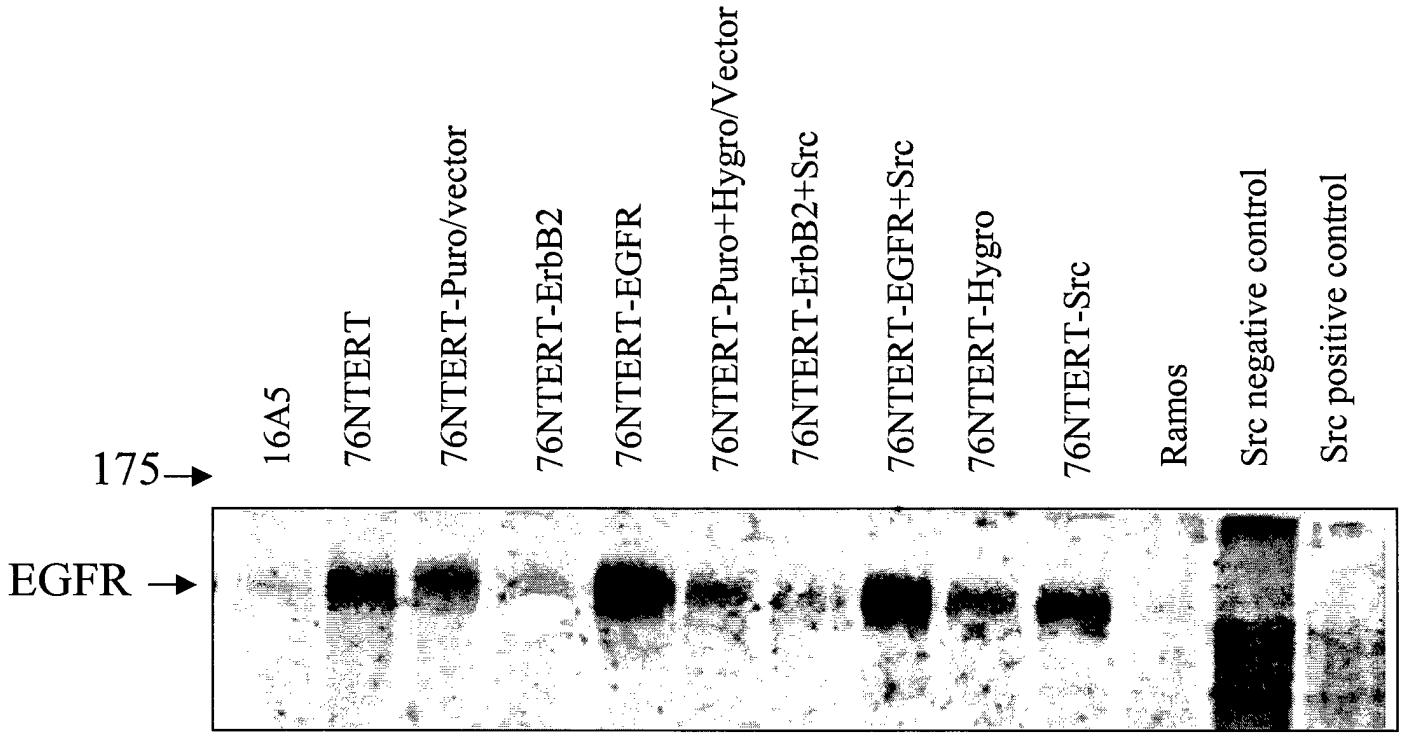


Fig. 6. Immunoblot analysis of EGFR overexpression in 76N-TERT transfectants. Cells were starved for 48 hrs by growth in EGF-deficient D3 medium and cell lysates were prepared in RIPA lysis buffer. 50µg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with with a cocktail of anti-EGFR monoclonal antibody (NeoMarkers). Ramos is a B-lymphoma line. Src-nagative and Src-positive controls are Src-/- and Src+/+ mouse embryonic fibroblasts. A relatively short exposure is shown to highlight the overexpression of EGFR; endogenous EGFR signals were present in all cell lines, except Ramos and fibroblast lines, upon longer exposure.

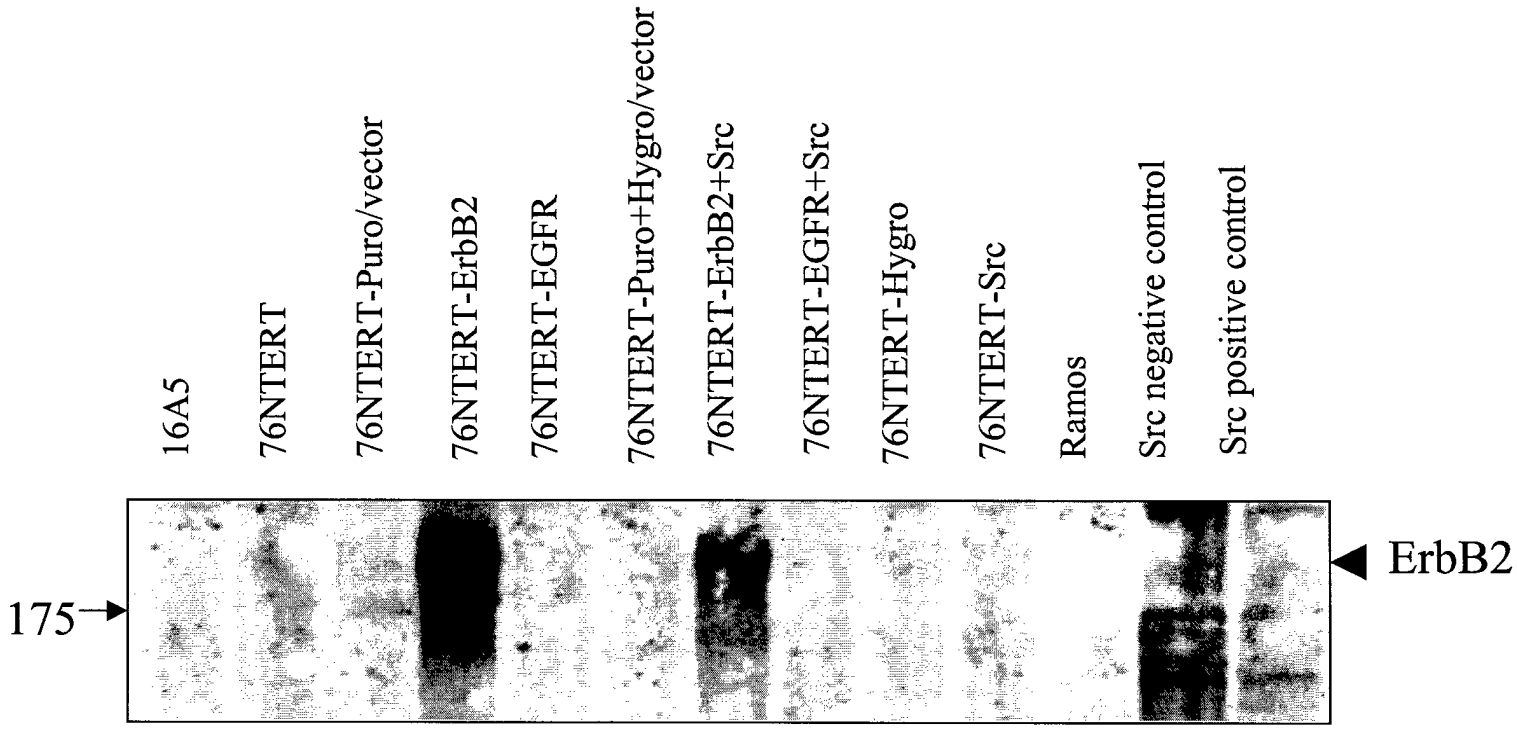
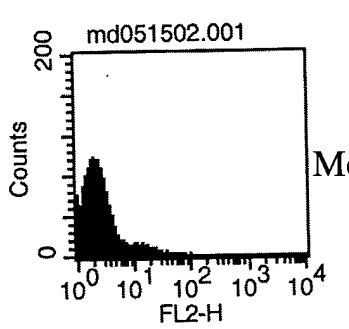


Fig. 7. Immunoblot analysis of ErbB2 overexpression in 76N-TERT transfectants. Cells were starved for 48 hrs by growth in EGF-deficient D3 medium and cell lysates were prepared in RIPA lysis buffer. 50μg aliquots of cell lysate protein were run on an 8% SDS PAGE gel and immunoblotted with with anti-ErbB2 monoclonal antibody (NeoMarkers). Ramos is a B-lymphoma line. Src-nagative and Src-positive controls are Src-/- and Src+/+ mouse embryonic fibroblasts. A relatively low exposure is shown to highlight the overexpression of ErbB2; endogenous ErbB2 signals were present in all cell lines, ecept for Ramos and fibroblast controls, upon longer exposure.

Fig. 8. Cell surface expression of EGFR or ErbB2 in mammary epithelial cell transfectants analyzed by FACS. Single cell suspensions were prepared by harvesting the cells with trypsin/EDTA. Live cells were stained with control antibody or with anti-EGFR or anti-ErbB2 antibodies for 1 hr, washed and incubated with the secondary antibody (PE-conjugated) for 45 min. FACS analysis was then carried out to determine the total cell surface levels of EGFR and ErbB2.

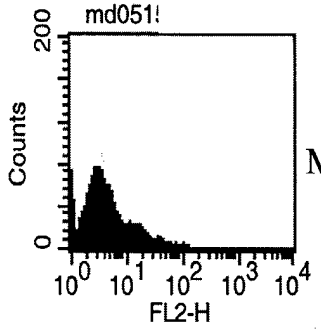
EGFR staining

76NTERT----Control



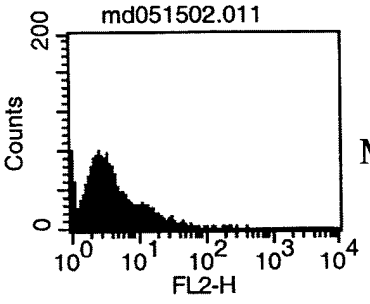
Median 2.17

76NTERT EGFR-----Control



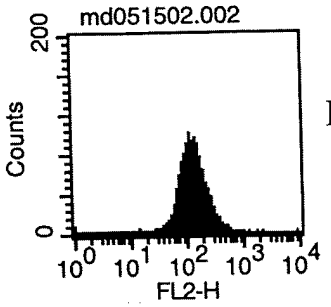
Median 3.62

76NTERT Src+EGFR



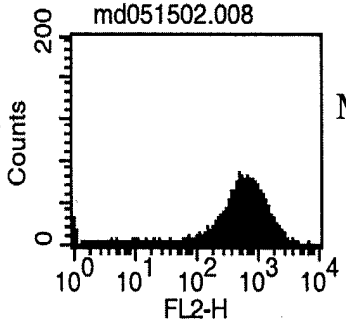
Median3.18

76NTERT----EGFR



Median 112.4

76NTERT EGFR-----EGFR



Median 528.03

76NTERT Src+EGFR

Median 276.32

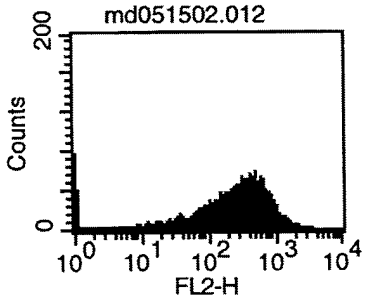
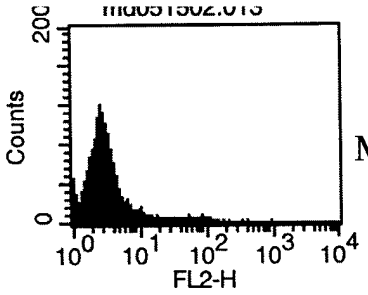


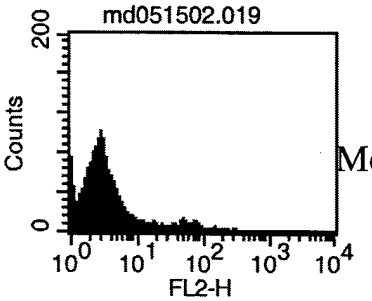
Fig. 8, Contd. ErbB2 staining

76NTERT----Control



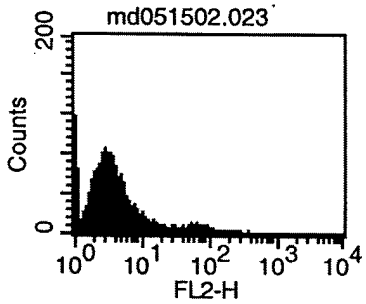
Median 2.74

76NTERT ErbB2----Control



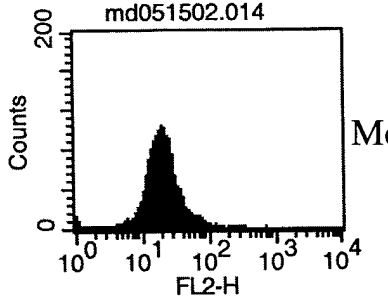
Median 2.79

76NTERT Src+ErbB2----Control



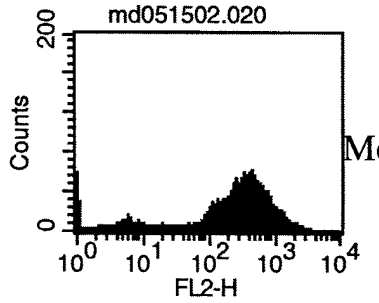
Median 3.28

76NTERT----ErbB2



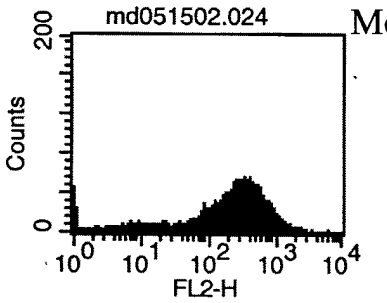
Median 18.6

76NTERT ErbB2----ErbB2



Median 310.5

76NTERT Src+ErbB2----ErbB2



Median 254.83

Fig. 9. Anchorage-independent growth of the human mammary epithelial cell transfectants. Single cell suspensions were prepared by harvesting the cells with trypsin/EDTA. The cells were then suspended in 0.35% agar in growth medium (DFCI-1 medium for transfectants and MEM-alpha for MCF7 cells) and plated on top of a bottom agar layer (0.5% agar). For each cell line, $3.3x10^4$ $1x10^5$ or $3x10^5$ cells were seeded in triplicate per 60 mm diameter dish. 0.5 ml of medium was added per dish and fresh medium was added every two days. Colonies were counted and photographed after 2 weeks. MCF-7 cell line was used as a positive control.

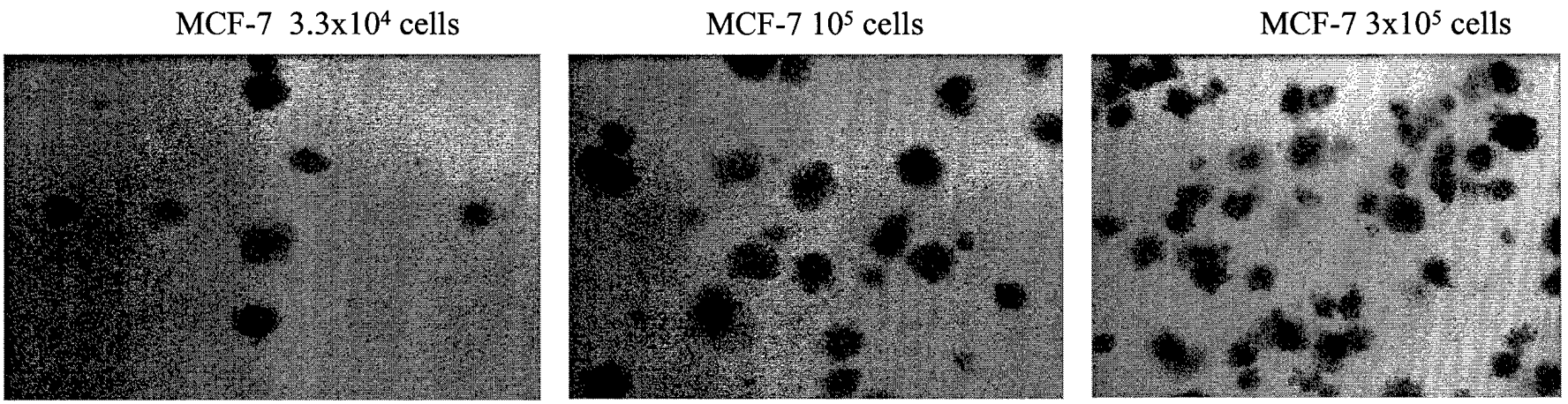
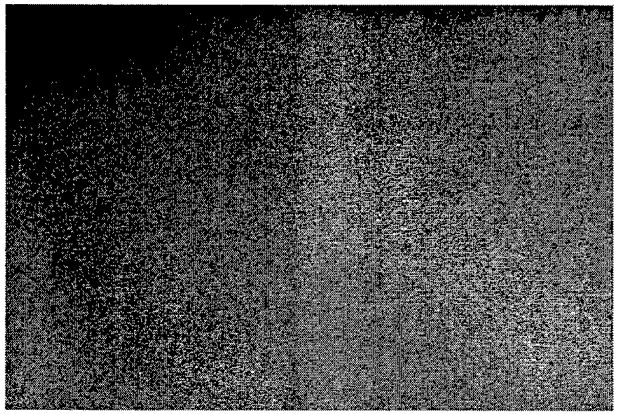
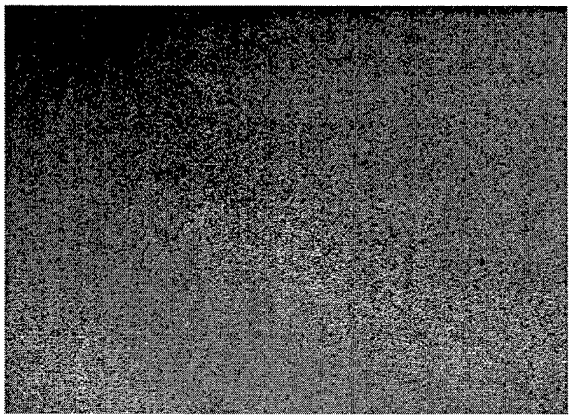


Fig. 9, Contd.

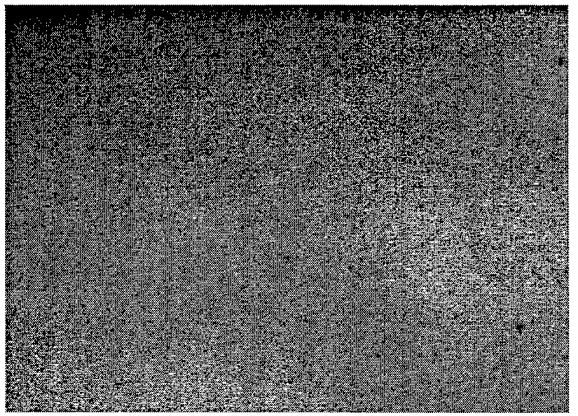
16A5-Puro/vector 3.3 x10^4 cells



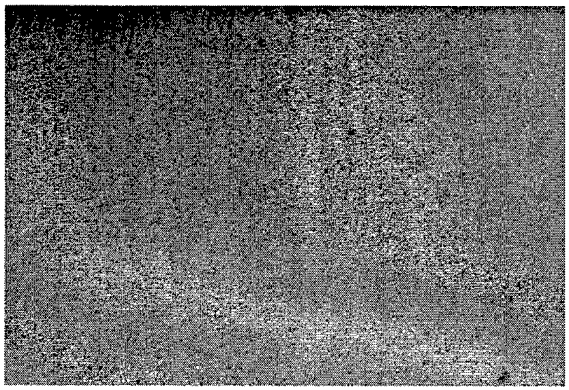
16A5-Puro/vector 10^5 cells



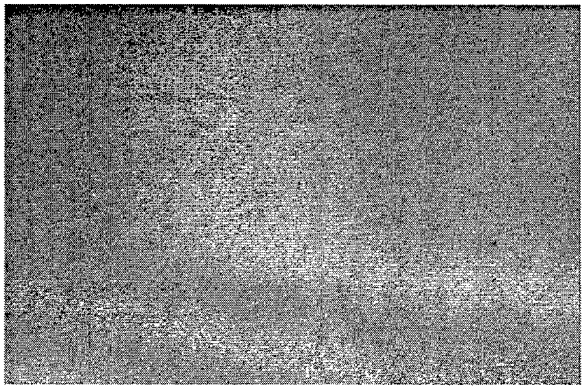
16A5-Puro/vector 3x10^5 cells



16A5-H 3x10^5 cells



16A5 3x10^5 cells



76NTERT 3x10^5 cells

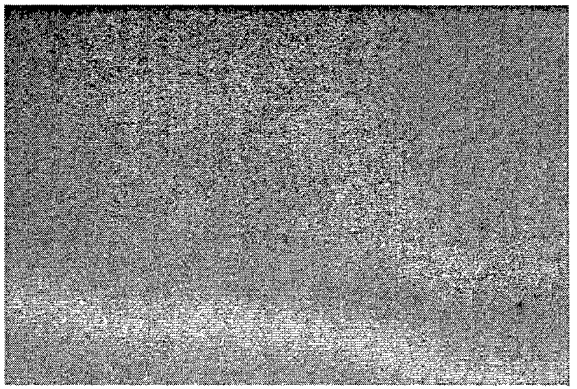
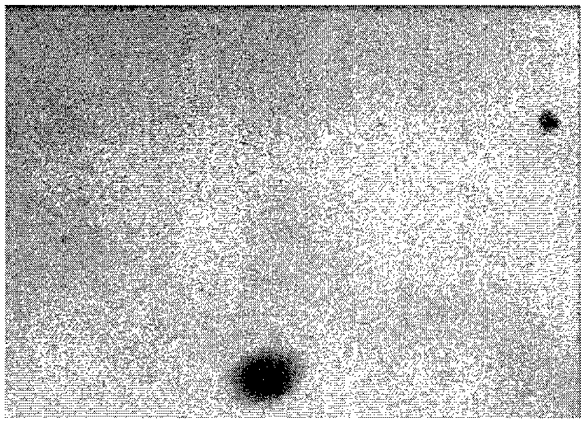
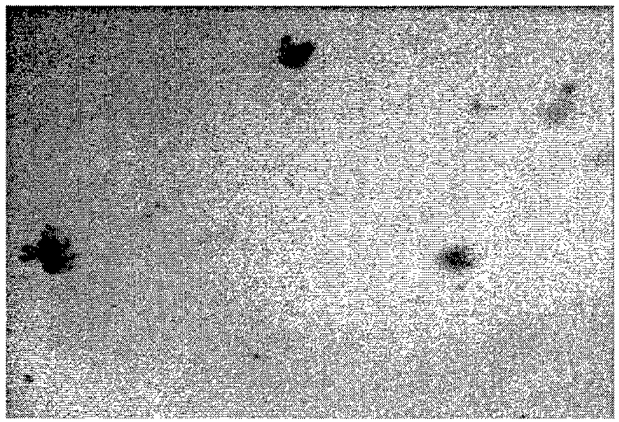


Fig. 9, Contd.

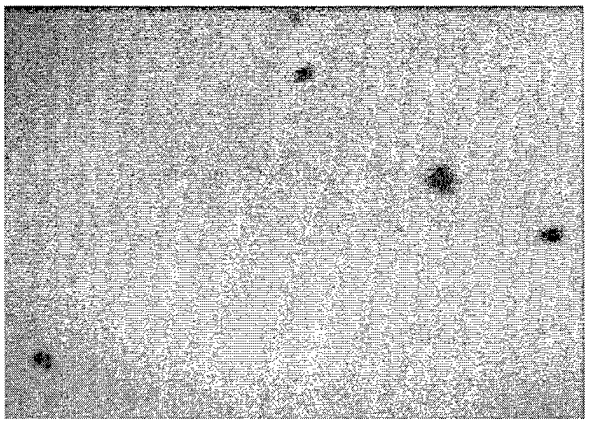
16A5-Src $3.3x10^4$ cells



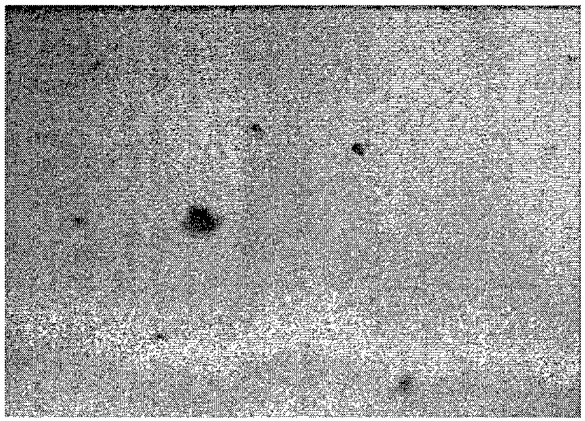
16A5-Src 10^5 cells



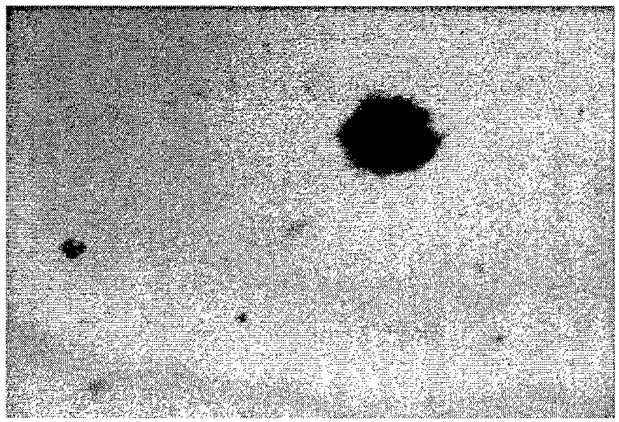
16A5-Src $3x10^5$ cells



16A5-EGFR $3.3x10^4$



16A5-EGFR 10^5 cells



16A5-EGFR $3x10^5$ cells

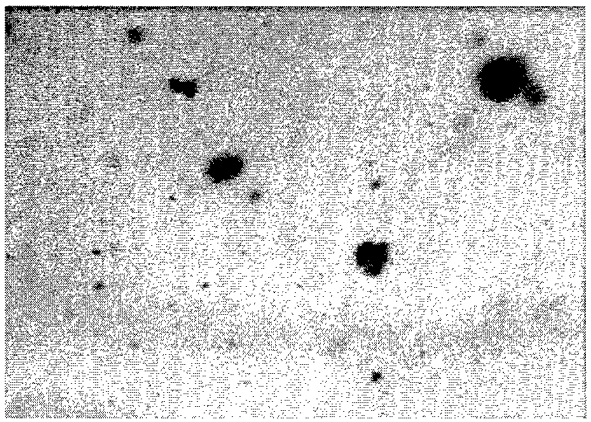
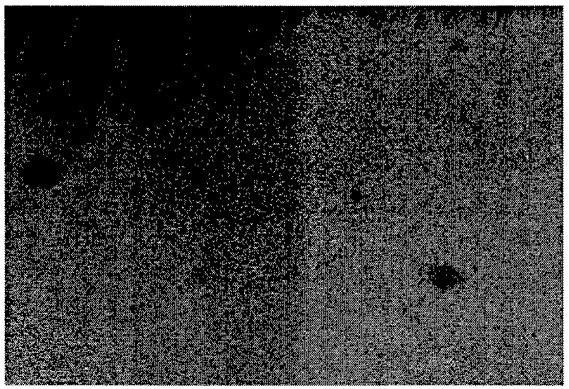
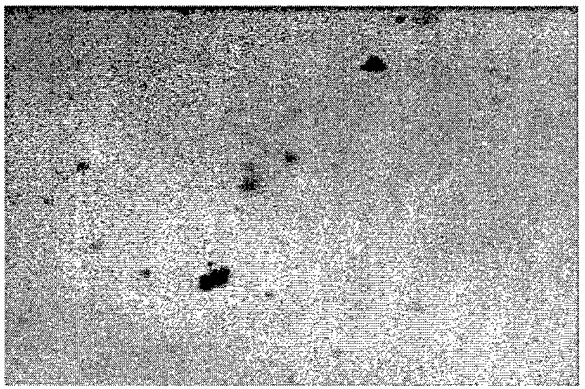


Fig. 9, Contd.

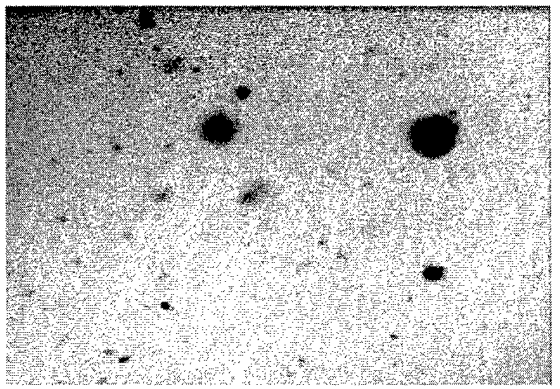
16A5- ErbB2 $3.3x10^4$ cells



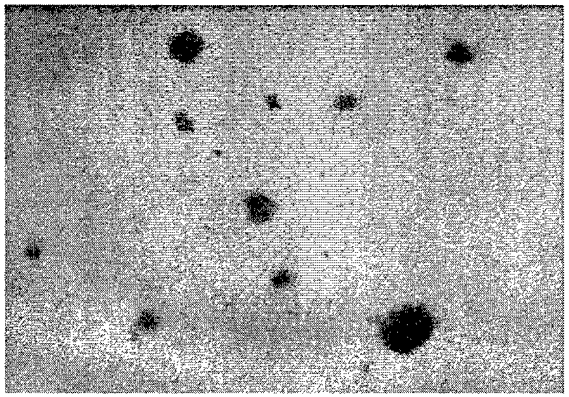
16A5- ErbB2 10^5 cells



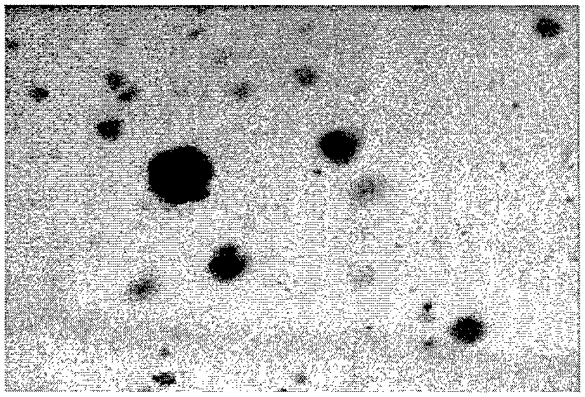
16A5- ErbB2 $3x10^5$ cells



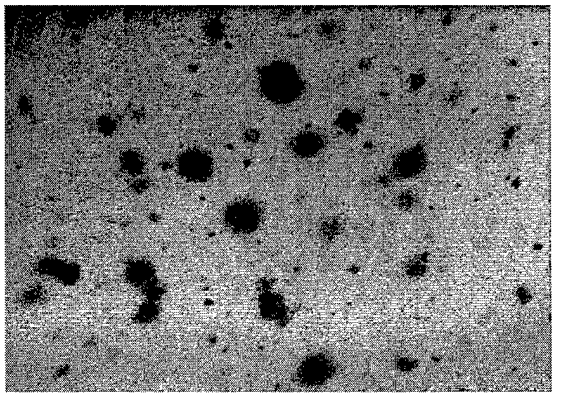
16A5-Erb+Src $3.3x10^4$ cells



16A5-Erb+Src 10^5 cells



16A5-ErbB2+Src $3x10^5$ cells



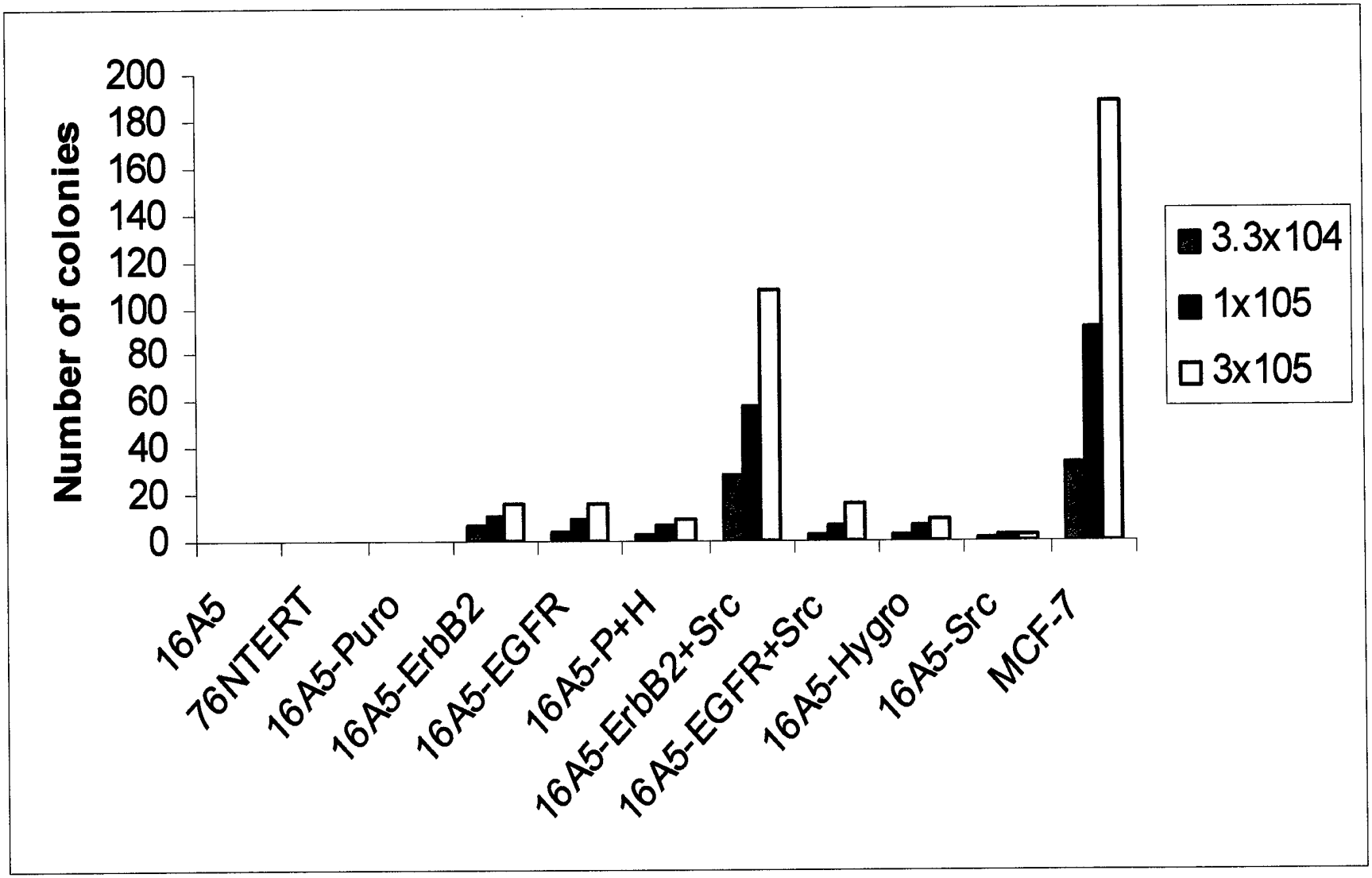


Fig. 10. Quantification of the anchorage-independent growth of mammary epithelial cell transfectants as determined by the number of colonies in the soft agar growth assay. Number of colonies were determined for each input cell number.

Fig. 11. **Growth of human mammary epithelial cell lines over-expressing various combinations of EGFR, ErbB2 and Src on reconstituted basement membrane (Matrigel).** Single cell suspensions were prepared by harvesting the cells with trypsin/EDTA. 0.75×10^6 cells in one ml of growth medium (DFCI-1 medium) were mixed with an equal volume of reconstituted basement membrane (BD Matrigel Matrix, Discovery Labware) and plated on top of a bottom layer of Matrigel. One ml of medium was added on top and changed every other day. The cells were photographed using the phase contrast optics on day 12.

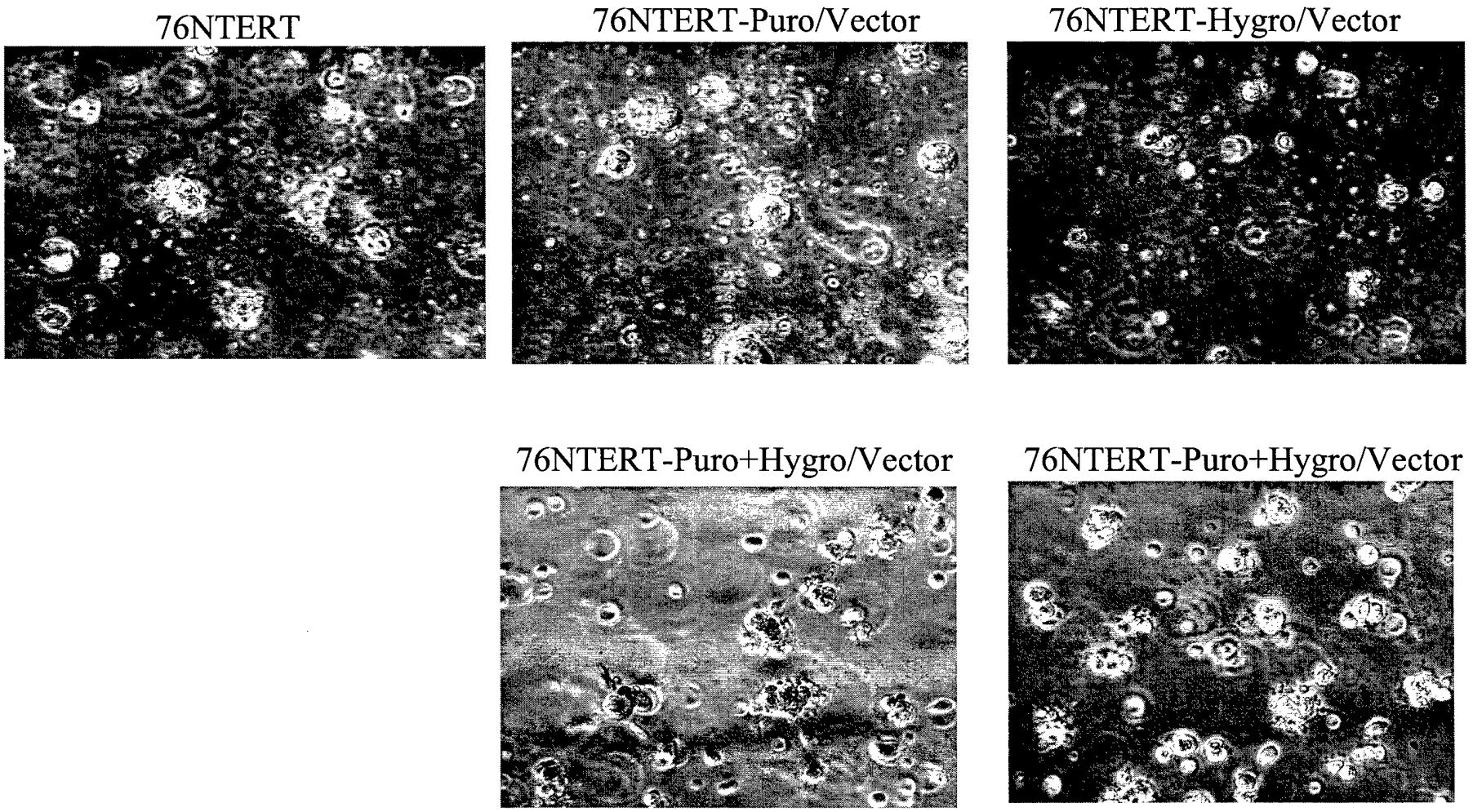
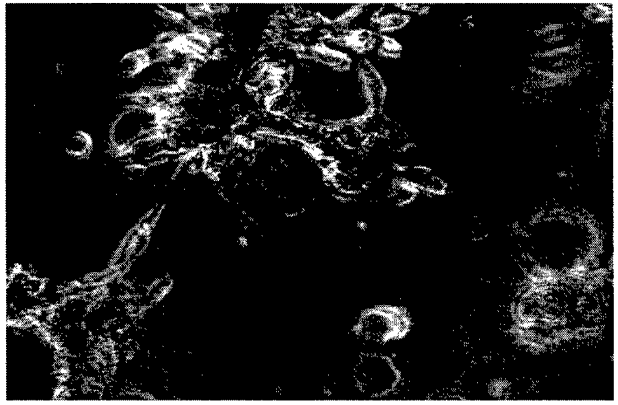


Fig. 11, Contd.

MDA-MB-231



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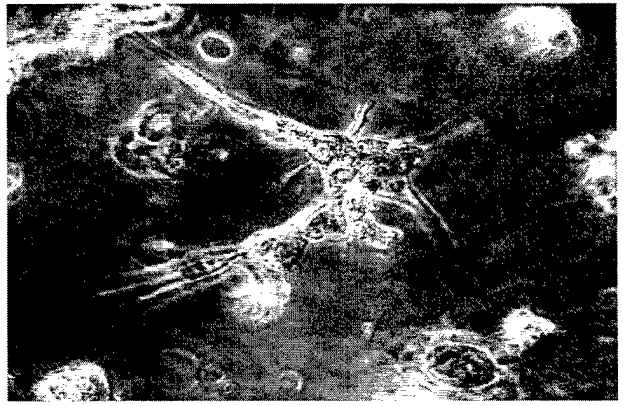
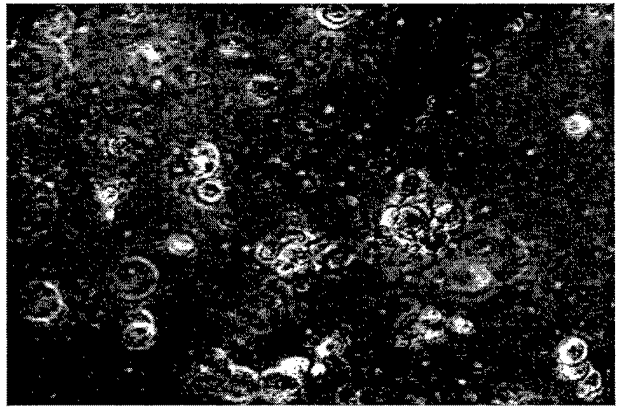


76NTERT-P-EGFR



76NTERT-P-EGFR

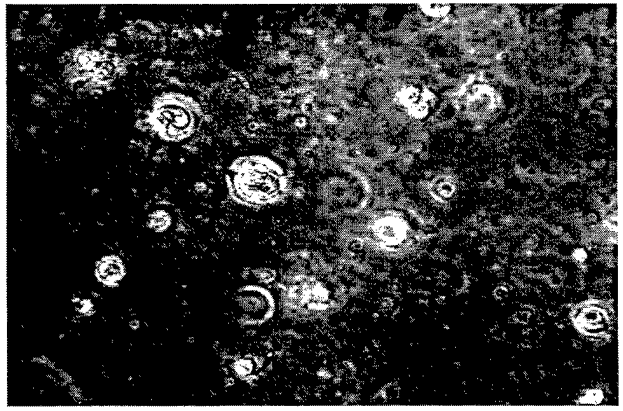
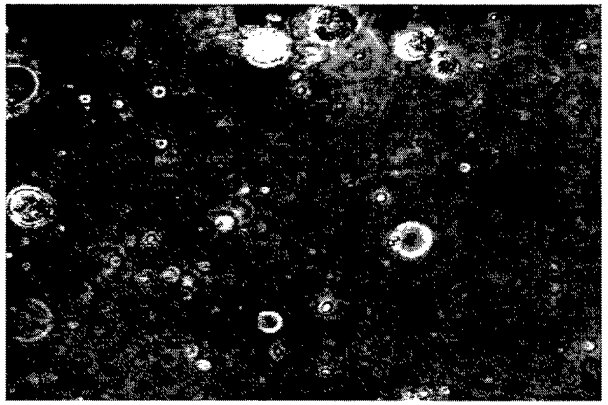
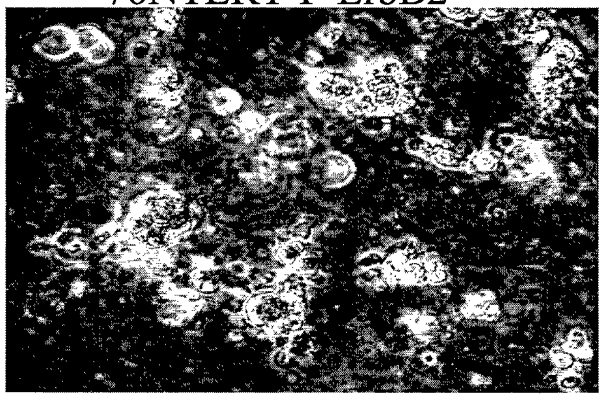


Fig. 11, Contd.

76NTERT-cSrc



76NTERT-P-ErbB2



76NTERT-P-ErbB2

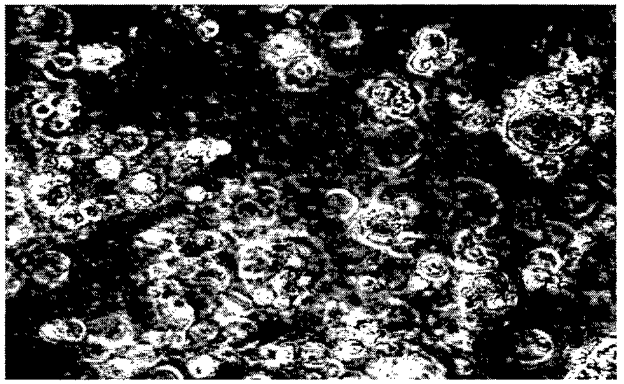
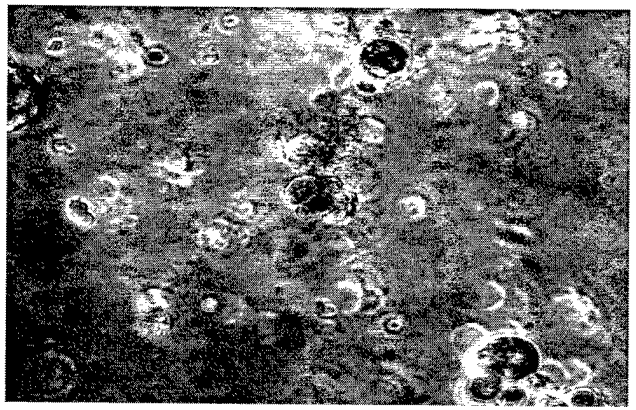
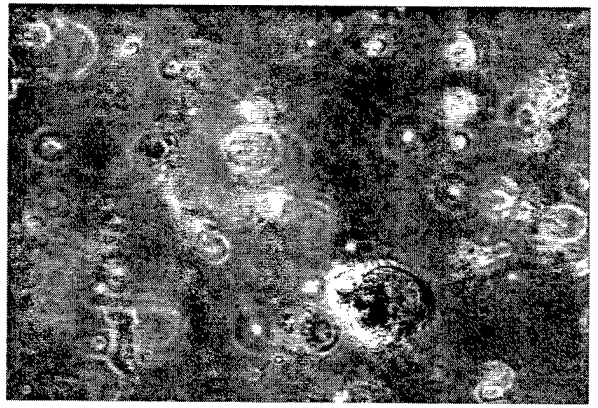


Fig. 11, Contd.

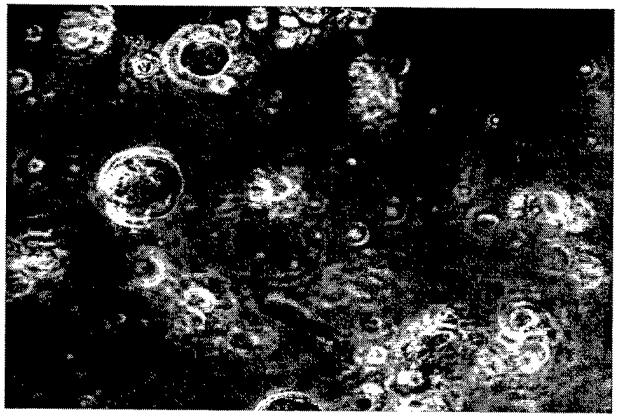
76NTERT-EGFR+cSrc



76NTERT-EGFR+cSrc



76NTERT-ErbB2+cSrc



76NTERT-ErbB2+cSrc

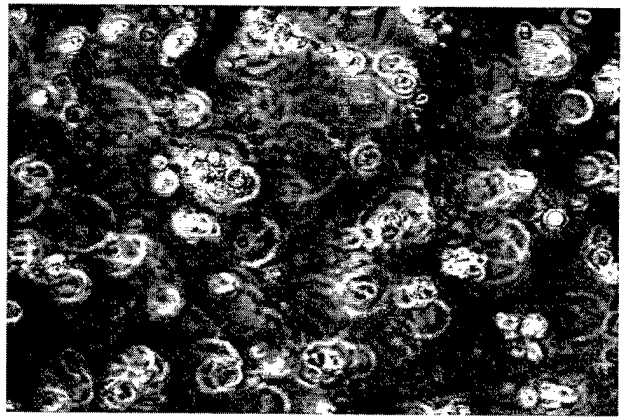


Fig. 12. Subcellular localization of EFGR, ErbB2 and Src in 76N-TERT transfectants as determined by immunofluorescence staining. Cells were grown on cover slips for 24hrs, starved for 48hrs in D3 medium, and then left unstimulated (EGF, o") or stimulated with 100ng/ml EGF for 30min (EGF 30"). The cells were fixed in paraformaldehyde for 20min, and stained with the primary antibodies anti-EGFR(528), anti-ErbB2(Ab-5) and anti-Src(327) for1hr, followed by staining with Texas red conjugated secondary antibody for 45min. Pictures were taken under oil immersion objective on a Nikon fluorescence microscope

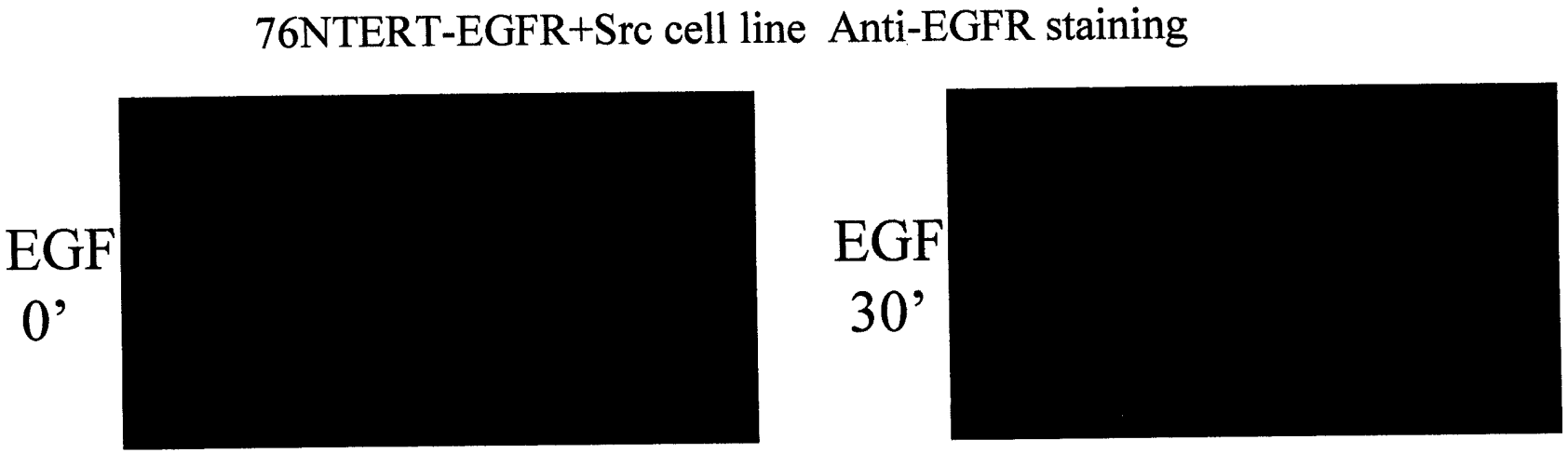
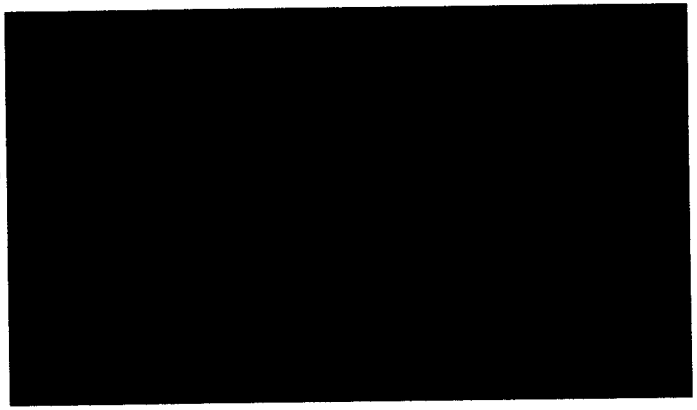


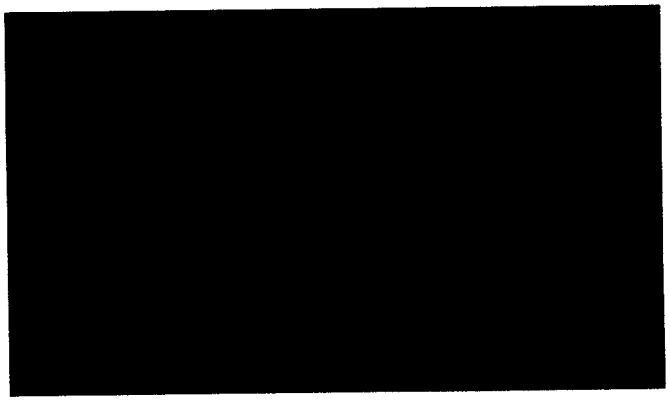
Fig. 12, Contd.

76NTERT-ErbB2+Src cell line, Anti-ErbB2 staining

EGF 0'

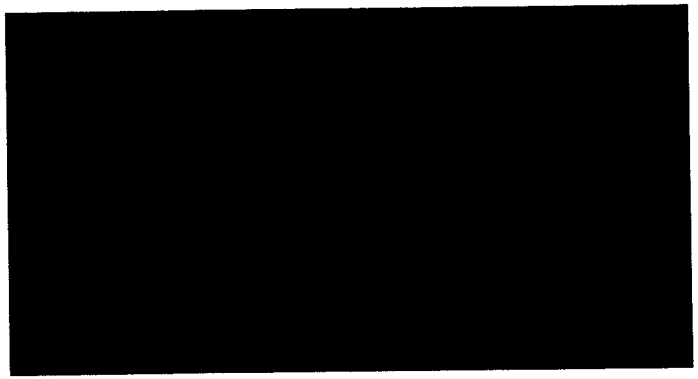


EGF 30'



76NTERT-ErbB2+Src cell line, Anti-Src staining

EGF 0'



EGF 30'

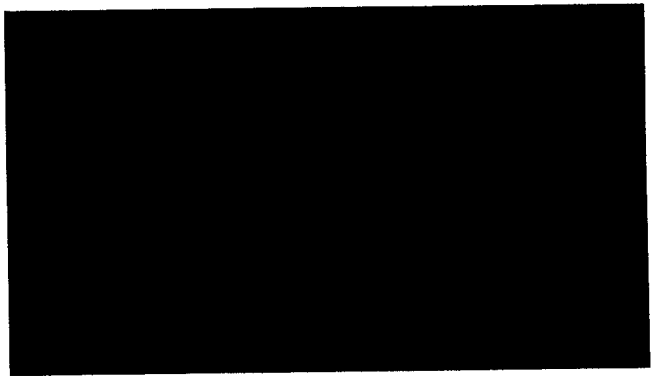


Table 1. List of Mammary Epithelial Cell Transfectants

Cell Lines	EGFR Expression	ErbB2 Expression	Src Expression
76NTERT-Puro/Vector			
76NTERT-EGFR	✔		
76NTERT-ErbB2		✔	
76NTERT-Puro+Hygro/Vector			
76NTERT-EGFR+Src	✔		✔
76NTERT-ErbB2+Src		✔	✔
76NTERT-Hygro/Vector			
76NTERT-Src			✔
76NTERT			
16A5-Puro/Vector			
16A5-EGFR	✔		
16A5-ErbB2		✔	
16A5-Puro+Hygro/Vector			
16A5-EGFR+Src	✔		
16A5-ErbB2+Src		✔	✔
16A5-Hygro/Vector			
16A5-Src			✔
16A5			
M2E6E7			
M2E6E7-Puro/Vector			
M2E6E7-EGFR			
M2E6E7-ErbB2		✔	
M2E6E7-Puro+Hygro/Vector			
M2E6E7-EGFR+Src			✔
M2E6E7-ErbB2+Src		✔	✔
M2E6E7-Hygro/Vector			
M2E6E7-Src			✔

Cbl mediated ubiquitination is an endosomal sorting signal for degradation of EGFR

Duan L*, Miura Y*, Dimri M, Majumder B, Dodge I, Lakkureddi A, Ghosh A, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Bowtell D, Gu H, Naramura M, Band V, Band H.

Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115

*These authors contributed equally)

Introduction

Epidermal growth factor receptor (EGFR or ErbB1) is a member of the ErbB family (ErbB1-4) of receptor tyrosine kinases that control cell proliferation, apoptosis, migration and differentiation during development and tissue homeostasis. A fundamental feature of ErbB receptor function is that ligand-induced dimerization promotes trans-phosphorylation of cytoplasmic tails, leading to the assembly of signaling protein complexes and activation of multiple enzymatic cascades. Notably, activated receptors undergo endocytosis via calthrin-coated vesicles **(Vieira AV, Lamaze C, Schmid SL. Control of EGF receptor signaling by clathrin-mediated endocytosis. Science. 1996 Dec 20;274(5295):2086-9.).** Endocytosed receptors go through a sorting process, either recycling back to the cell surface for continued ligand binding and signaling or proceeding to the lysosome for degradation **(Herbst JJ, Opresko LK, Walsh BJ, Lauffenburger DA, Wiley HS. Regulation of postendocytic trafficking of the epidermal growth factor receptor through endosomal retention. J Biol Chem. 1994 Apr 29;269(17):12865-73. 1,2,3,4);** the efficiency of this sorting process is a key determinant of ErbB signaling potency (6, **Waterman H, Sabanai I, Geiger B, Yarden Y. Alternative intracellular routing of ErbB receptors may determine signaling potency. J Biol Chem. 1998 May 29;273(22):13819-27).**. For example, the EGF-activated EGFR is predominantly delivered to lysosomes, whereas heregulin-activated ErbB2 primarily recycles, accounting for the signaling superiority of ErbB2

We, and others, have identified the Cbl proto-oncoprotein as a critical regulator of this process. Cbl is an E3 ubiquitin ligase that selectively interacts with activated EGFR through its tyrosine kinase-binding (TKB) domain (18), which binds to EGFR pY1045 (14). The Cbl RING finger-

associated ubiquitin conjugating enzymes mediate ubiquitin transfer to EGFR (16, 17). The crucial role of ubiquitin as an internalization signal for yeast membrane receptors identified through genetic means has been established (7,8) and recently extended to mammalian cell surface receptors (9,10,11). Overexpression of dominant negative Cbl mutants blocks ubiquitination of EGFR that correlates with retarded degradation (22;23;24). However, it is not known at what step (internalization versus lysosomal sorting) the Cbl-mediated ubiquitination of EGFR is required and whether it is essential for lysosomal degradation of EGFR.

In the present study, we have used Cbl deficient mouse fibroblast cells and Chinese Hamster Ovary cells conditionally-defective for ubiquitination to address these issues. We demonstrate that Cbl is the major regulator of EGFR ubiquitination and the ubiquitination of EGFR is dispensable for its initial endocytosis but is required for its efficient delivery from early endosome to lysosome for degradation.

Experimental Procedures

Material

Biotinylated EGF Alexa-Fluo-488 streptavidin complex and Alexa-Fluo-488 conjugated transferrin were obtained from Molecular probes (Molecular Probes Inc, OR). Opti-MEM I Reduced Serum Media was acquired from GIBCO BRL (LIFE TECHNOLOGIES, MD). Fugene was obtained from Roche (Roche Molecular Biochemicals, IN).

Cell lines

CHO-Ts20 and CHO-E36 cells (Generously provided by Ger j. Strous, University Medical Center Utrecht, Heidelberglaan 100, Utrecht, The Netherlands)(28) were transfected with a pCDNA3 construct containing the full length human Epidermal Growth Factor Receptor (EGFR) cDNA sequence (22) using Fugene method (). Cbl $^{+/+}$ and Cbl –/– mouse embryonic fibroblast cells () were derived from wild type and Cbl knockout mice (). Both cell lines were stably transfected with full length human EGFR by retroviral transfection using pMSCV-pac plasmid constructs as described **(Bonita, D. P., Miyake, S., Lupher, M. L., Jr., Langdon, W. Y., and Band, H. (1997)** ***Mol. Cell. Biol.*** **17, 4597-4610)**. Stable geneticin or puromycin resistant transfectants were selected after screening with anti-EGFR antibodies by FACS and immunoblotting. CHO Cells were grown in Eagle's minimal essencial medium (MEM; Life technologies, Inc.) supplemented with 10% FBS, penicillin and streptomycin, and 0.5 mg/ml geneticin. Mouse fibroblast cells were were grown in MEM supplemented with 10% FBS, penicillin and streptomycin, and 2.5 µg/ml puromycin. For experiment the cells were grown on 100 mm plates and used at 80% confluence.

Antibodies

The purified anti-EGF-R monoclonal antibody (EGFR 528), anti-EGFR rabbit polyclonal antibody (EGFR 1005; sc-03), were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The anti-Tyr(P) mAB 4G10 (IgG2a; Ref 39) was the kind gift of Brian Druker (Oregon Health Sciences University, Portland, OR).The anti-ubiquitin mAB P4G7 ascites (IgG1) was obtained from Covance (Covance Research Product Inc, PA). The rabbit anti-Lamp1 serum 931B (Ref JBC263, 18911, 1998) was a generous gift

from Fukuda (The Burnham Institute, CA). Cy3-conjugated goat-anti-mouse, Cy2- conjugated goat-anti-rabbit.antibodies were purchased from Jackson (Jackson ImmunoResearch Laboratories Inc, PA). The rat anti-lamp1 monoclonal antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Transfection, EGF Stimulation, and Preparation of Lysates of Mammalian Cells Transfections were performed using Fugene method, cells were seeded into 100-mm tissue culture dishes 12 to 16 h prior to transfection, such that the cells were 10 to 20% confluent at the time of transfection. Plasmid DNA was mixed with Fugene at a ratio of 2.5 ul fugene/ug DNA in 60ul Opti-MEM and precipitated at room temperature for 15 to 25 minutes before it was added into the culture medium for transfection. Input doses of the cytomegalovirus promoter, present in the pCDNA3 constructs, were equalized within experiments using pcDNA3 vector. The amounts of input DNA are indicated in the figure legends. Culture media were replaced at 14-18 h following the addition of DNA precipitates. For transient transfection, Cells were harvested at 48 h following the addition of DNA precipitates. For stable transfection, cells were selected after 48 h as described above. For EGF stimulation, cells were placed in starvation medium (growth medium containing 0.5% FBS) for 4-6 h and then were incubated for the indicated lengths of time with purified murine EGF (Sigma) at the indicated concentration. The cells were rinsed with ice-cold PBS and lysed using Triton X-100 lysis buffer (50 mM Tris, (pH 7.5), 150 mM sodium chloride, 0.5% Triton X-100 (Fluka), 1 mM phenylmethylsulfonyl fluoride, 0.07 trypsin inhibitor units aprotinin/ml, and 1 μg/ml each of leupeptin, pepstatin, antipain, and chymostatin) as described (26). The protein concentration of

lysates was determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard.

Immunoprecipitation and Immunoblotting— The procedures for immunoprecipitation and immunoblotting were previously described (25). Amounts of lysate protein and the antibodies used are indicated in the relevant figure legends. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA). Immunodetection was performed using horseradish peroxidase-conjugated protein A or goat-anti-mouse antibody (Cappel/Organon Teknika Corp., West Chester, PA) and the Renaissance Western blot Chemiluminescence Reagent Plus kit (NEN Life Science Products). Where indicated, membranes were stripped and reprobed as described previously (25)

Fixed Cell Immunostaining and confocal Microscopy—Cells were seeded onto sterile glass coverslips in 6-well cell culture plates. 2 days after attachment, coverslips were incubated for 4-6 h in starvation medium and then were harvested at the indicated time points after stimulation with EGF as detailed above. After washing in ice-cold phosphate-buffered saline (PBS), coverslips were held in 4% formaldehyde in PBS for 20min. Formaldehyde was removed, and the cells were immunostained. All further processing was carried out at room temperature. For immunostaining, cells were incubated in blocking solution (1% bovine serum albumin and 0.02% saponin in PBS, filtered) for 15 min. The coverslips were rinsed briefly in

PBS, incubated in a solution of the appropriate primary antibody (4 μg/ml anti-EGF-R 528, 1:200 dilution of anti-lamp1 serum in blocking solution) for 30 min, and subjected to five 5-min blocking solution washes. Cells were then incubated with goat anti-(mouse IgG (H+L)), F(ab')2-Cy3 at 1: 300 dilution, rabbit IgG, F(ab')2-Cy2 in blocking solution. Following five 5-min washes in PBS, coverslips were mounted in Immunomount () on glass slides. Confocal microscopy were carried out by using the Leica TCS-NT confocal laser scanning microscope fitted with krypton and argon lasers as described before (29)

EGF Receptor internalization assay –Cells were plated on 10cm tissue culture dishes and grown for 48 hours till the cells reached about 70-80% confluence. Following serum starvation as described above, the cells were incubated with 100 ng/ml Alexa-Fluo 488-conjugated EGF at 4°C for 30 minutes, then washed with PBS for 3 times, returned back to growing temperature to let EGFR be internalized. At the indicated time points, the cells were placed on ice to stop internalization and rinsed with cold PBS for three times, subjected to acid wash (0.2M acetic acid, 0.5M NaCl , PH 2.8) for 5 minutes, then washed 3 times with PBS to remove the uninternalized EGF. The cells were detached from tissue culture dishes using 10 mM EDTA in PBS, and washed with FACS buffer (2% FBS and 0.01% sodium azide in PBS). After three washes, cells were resuspended in FACS buffer and fixed by addition of an equal volume of 4% formaldehyde. Fluorescence emission from the internalized EGF was detected by flow cytometry. Background binding was determined by acid wash of the cells without allowing internalization. Non-acid-washed cells were used to calculate the percentage of internalization. Flow cytometry, data collection, and analysis

were performed on a FACSort machine using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Downregulation of EGFR–Downregulation of EGFR from cell surface is performed as being described before (22)

Results

Characterization of Cbl $^{+/+}$ **and** Cbl –/–**mouse embryonic fibroblast cells**—Cbl+/+ and Cbl –/–fibroblast cells were derived from Cbl $^{+/+}$ amd Cbl –/–mouse embryo (). Western blot was conducted by using anti-Cbl and anti-Cbl-b antibodies to detect Cbl and Cbl-b in total cell lysates. Cbl was detected in wild type cells but not in Cbl –/–cells (Fig1a).

Ubiquitination of EGFR in Cbl –/–**MEF cells is radically impaired which correlates with retarded degradation of the receptors** — Cbl has been shown to regulate the ubiquitination of EGFR. However, it is not known weather it is essential for the ubiquitination and degradation of EGFR under physiological conditions. By Comparing Cbl $^{+/+}$ and Cbl –/– MEF cells, we would be able to determine the role of endogenous Cbl in the downregulation of EGFR. Cells were stably transfected with human EGFR and stimulated with EGF, at the indicated time points, cells were harvested and EGF-receptors were immunoprecipitated from total cell lysates and western blot was performed to detect ubiquitination. EGFR was ubiquitinated after stimulation in Cbl $^{+/+}$ cells. Conversely, EGFR ubiquitination was reduced to approximately 20-30 % level in Cbl –

/–cells compared to that in Cbl $^{+/+}$ cells, phosphorylation of EGFR after stimulation is not decreased (Fig 1b). Indicating Cbl as the major ubiquitin ligase for EGFR in those cells. In accordance with the impaired ubiquitination, the degradation of EGFR is retarded in Cbl –/–MEF cells (Fig 1c). Cbl-/- cells were reconstituted with HA tagged Cbl by stable infection with a retroviral vector. The reconstituted cells have 2.7 times more Cbl compared with wild type cells (Fig 2a). Ubiquitination of EGFR is completely restored in the HA-Cbl reconstituted Cbl –/– cells (Fig 2b). The degradation of the receptors, is accelerated to the same level as it is in the Cbl $^{+/+}$ cells (Fig2c).

Internalization of EGFR is not affected while downregulation of EGFR is decreased in Cbl-/- MEF cells—Cbl has been implicated being involved in the endocytosis of EGFR (). Since the degradation of EGFR is impaired in Cbl –/– cells, we wanted to ask weather the receptor degradation is affected at the early stage of endocytosis by impeded internalization. To measure EGFR internalization in those cells, ligand-initiated internalization was induced with Alexa-Fluo488-conjugated EGF, fluorescence signals from internalized EGF were detected by flow cytometry. Internalization was measured in a short period of time (10 minutes) to reduce the error generated by the recycling EGF receptors. Internalization velocity of EGFR is not different in Cbl –/– cells from that in Cbl $^{+/+}$ cells (Fig 3a, left panel, P ≤. Unimpaired internalization of EGFR in both cell lines were further confirmed by fluorescence staining of EGFR in the endocytosed vesicles after EGF stimulation (Fig 3b). Cbl-/- cells were reconstituted with nearly 3 times as much HA-Cbl as it does in the wild type cells. Internalization of

EGFR in those cells is not increased compared to Cbl –/– cells that was reconstituted with vector. Despite of unaffected internalization, downregulation of EGFR from the cell surface is significantly decreased in Cbl –/– cells (3c, left panel). The downregulation of EGFR regulated by Cbl is further reinforced by reconstituting Cbl –/– cells with HA-Cbl, that greatly enhanced downregulation of EGFR from cell surface (Fig 3c, right panel). Combining unaffected internalization of EGFR with the decreased downregulation of EGFR in Cbl –/– cells, we conclude that recycling of EGFR back to cell surface is increased in Cbl –/– cells.

Degradation of EGFR is retarded when ubiqutination is blocked — Since ubiquitination of EGFR is not completely blocked in Cbl –/– MEF cells, we used another system to explore the role of ubiqutination in the degradation of EGFR. Ts20 cells has a temperature-sensitive E1 (ubiquitin-activating) enzyme which will lose its activity when cells are grown at the non-permissive temperature (27). Therefore, the ubiquitination machinery will be halted under such a condition. E36 cells do not have such a defect so that they were used as control. Ts20 cells and E36 cell lines were stably transfected with human EGFR and were grown at the permissive temperature (30°C) versus the non-permissive temperature (42°C) . Cells were stimulated with EGF after starvation. EGFR in both lines of cells were rapidly ubiquitinated following stimulation when being cultured at the permissive temperature (Fig 4a, b, left panels). Ubiquitination of EGFR was blocked in Ts20 cells (Fig 4a, right panel) when the cells were cultured at the non-permissive temperature for 4 hours. In contrast, ubiquitination of EGFR was almost the same at either the permissive temperature (Fig 4b, left panel) or non-permissive temperature (Fig 4b, right panel) in E36 cells

Concurrently, EGFR underwent degradation after stimulation that correlated with ubiquitination under the permissive temperature condition. On the other hand, degradation of EGFR was considerably retarded in Ts20 cells when ubiquitination was blocked at the non-permissive temperature (Fig 4c). That is not seen in E36 cells, in which both ubiquitination and degradation of EGFR was not affected under the non-permissive temperature condition (Fig 4d).

Ubiquitination regulates endosomal sorting of EGFR but not internalization of EGFR — In Ts20 cell lines, the internalization rate of EGFR was not obstructed when cells were grown at the non-permissive temperature to block ubiquitination machinery, compared to the permissive temperature condition ($P<0.05$) (Fig 5a). The same results were obtained in control E36 cells (Fig 5b). These results indicate that ubiquitination is a dispensable factor for the internalization of EGFR. The process of ligand-initiated endocytosis of EGFR was tracked by fluorescence staining of EGFR after EGF stimulation in the both cell lines. Under the permissive temperature condition, in both Ts20 and E36 cell lines, after stimulation with EGF, EGFR underwent rapid internalization and distributed in endosomal compartments in the cells. At the permissive temperature, in both cell lines, those vesicles initially showed peripherally-distributed pattern (Fig 6A, b1; Fig 7A, b1) at 5 minutes and gradually moved to the center of cells and clustered at 30 minutes (Fig 6A, c1; Fig 7A, c1). At the non-permissive temperature, the same pattern was still seen in E36 cells (Fig 6B, e2, f2). In contrast, the vesicles of EGFR showed a delayed peripherally-distributed pattern in Ts20 cells at 30 minutes after EGF stimulation under the non-

permissive temperature conditon (Fig 7B, f1). To characterize those endosomal compartments, those cells were either loaded with fluorescence-conjugated transferrin, a early/recycling endosome marker, or co-stained anti-LAMP-1, a late endosome marker. Then analyzed with confocal microscopy for their co-localization with EGFR. At the permissive temperature, in both cell lines, at 5 minutes after EGF stimulation, the internalized EGFR displayed in peripherally-distributed vesicles that co-localized with transferring (Fig 6A: b3, b4; Fig 7A: b3, b4). At 30 minutes after stimulation, EGFR distributed in the clustered vesicles at the center of cells, dissociated from transferring enriched vesicles (Fig 6A: c3, c4; Fig 7A: c3, c4), became co-localizing with LAMP-1 (Fig 8A: b3, b4; Fig 8B e3,e4), indicating that internalized EGFR underwent endocytic transport from early endosome to late endosome. When Ts20 cells were grown at the non-permissive temperature to disrupt the ubiquitination machinery, majority of the internalized EGFR still distributed in the peripherally-distributed vesicles that remained colocalizing with transferrin (Fig 7B: f3, f4), but not with Lamp1(Fig 8B: f3, f4) at 30 minutes after stimulation. While the control E36 cells, in which ubiquitination was not blocked under such conditions, exhibited the same pattern of vesicle distribution and colocalization as shown under the permissive temperature conditions (Fig 6B: f3, f4; Fig 8A: e3, e4). Those results indicate that ubiquitination regulate endosomal sorting of EGFR that starts at very early step after the internalization.

Discussion

It is completely unknown how the internalization of EGFR is regulated on the molecular basis of the receptors in the ligand-initiated endocytosis.

Although several common endocytic motifs that are conserved in the molecule, such as tyrosine-based AP-2 binding motif **(Nesterov A, Wiley HS, Gill GN. Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. Proc Natl Acad Sci U S A. 1995 Sep 12;92(19):8719-23.)** and dileucine-based **motif (Kil SJ, Hobert M, Carlin C. A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand-receptor complexes to lysosomes. J Biol Chem. 1999 Jan 29;274(5):3141-50.)**, are implicated in the regulation of internalization, none of them have been shown to be essential for the internalization of EGFR by either truncation or point mutation analysis (). Seeing that ubiquitination controls receptor internalization in many systems, such as alfa-factor receptor in yeast (7;8) and growth hormone receptor in mammalian cells (9). Ubiquitination of EGFR, which occurs at the cell membrane **(Stang E, Johannessen LE, Knardal SL, Madshus IH. Polyubiquitination of the epidermal growth factor receptor occurs at the plasma membrane upon ligand-induced activation. J Biol Chem. 2000 May 5;275(18):13940-7.)**, may play a role in the receptor internalization. Besides, Cbl is implicated being involved in the internalization of EGFR as an adaptor by enrolling CIN85 and endophilin to form a complex with the receptors (37) and regulating monoubiquitination of CIN85 **(Haglund K, Shimokawa N, Szymkiewicz I, Dikic I. Cbl-directed monoubiquitination of CIN85 is involved in regulation of ligand-induced degradation of EGF receptors. Proc Natl Acad Sci U S A. 2002 Sep 17;99(19):12191-6.)**. However, in the present study, the internalization rate of EGFR is not blocked in Cbl –/– cells, in spite of the impaired ubiquitination and degradation of EGFR. Moreover, internalization of EGFR is not affected when ubiquitination is blocked in Ts20 cells. We conclude that neither Cbl nor ubiquitination is essential for the internalization of EGFR in those cells. Those results are consistent with

previous publication in that dominant negative mutant of Cbl blocked ubiquitination and degradation but not affect internalization of EGFR (22). They are conflicting to the recent publication in that a single mutation of 1045 tyrosine on EGFR that abolishes the docking site for Cbl blocked internalization of EGFR (), but compatible to previous publication in that a c-terminal deletion of EGFR from 993 to 1186 which contains the docking site for Cbl did not affect the internalization of EGFR (). We reason that in some cell system Cbl might be essential but in others it is just one of the redundant mechanisms that control the internalization of EGFR. Unfortunately, we are not able to evaluate the role of the other Cbl family members in the internalization of EGFR in Cbl –/– cells. Even though Cbl-b expression level is very low in those cells, we do not have available antibody to detect Cbl-C in those cells.

Up till now, Cbl family is the only type of E3 ubiquitin ligase found to regulate the ubiquitination of EGFR. By using Cbl knockout MEF cells in which the ubiquitination is regulated by endogenous E3 ubiquitin ligases, we can determine weather Cbl is essential in the ligand initiated downregulation of EGFR. Ubiquitination of of EGFR is dramatically blocked in Cbl –/– cells, suggests that Cbl be the major ubiquitin ligase that regulates the ubiquitination of EGFR in those cells. Along with using conditional ubiquitin defective cells to block ubiquitination. We observed that degradation of EGFR was delayed whenever ubiquitination of EGFR is blocked either in the absence of Cbl in Cbl-/- MEF cells or in the presence of Cbl in Ts20 cells. That suggests that Cbl regulated degradation of EGFR be dependent on its ubiquitin ligase activity on the receptors rather than other mechanisms mediated by itself. Interestingly, blocking ubiquitination of

EGFR only retarded, but not fully blocked degradation of the receptors in both systems. It is very likely that ubiquitination is only a facilitator rather than an indispensable factor for EGFR to be degraded. Though we cannot completely rule out the possibility that the remnant ubiquitination in those cells might be an enough signal for the receptors to be eventually degraded. One of the other possible mechanisms in the regulation of EGFR degradation is the dileusine-based motif which can be recognized by the degradation machinery. The dileusine motif has been shown to control the sorting of EGF receptors from early endosomes to late endosomes (). The ongoing study is trying to explore the regulatory relationship between the ubiquitination of EGFR and dileusine-based sorting pathway.

It appears that the crucial role of ubiquitination in the degradation of EGFR comes into being after the receptors are internalized. Many lines of evidence suggested that ubiquitin be a tag for EGFR to be picked up by endosomal sorting proteins that have specific ubiquitin interacting motif and then delivered from endosome to lysosome. The hepatocyte growth factor receptor substrate (Hrs) has a ubiquitin interacting motif which binds ubiquitinated proteins *(**Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P, Di Fiore PP.** A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature. 2002 Mar 28;416(6879):451-5. **Shih SC, Katzmann DJ, Schnell JD, Sutanto M, Emr SD, Hicke L.** Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. Nat Cell Biol. 2002 May;4(5):389-93. **Raiborg C, Bache KG, Gillooly DJ, Madshus IH, Stang E, Stenmark H.** Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat Cell Biol. 2002 May;4(5):394-8.)* and a FYVE domain that locates it to early endosome *(**Komada M, Soriano P.** Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. Genes Dev. 1999 Jun 1;13(11):1475-85.).* A

truncated form of Hrs that does not contain intact UIM impaired degradation of EGFR in *Drosophila* by affecting endosome membrane invagination and formation of multivesicular bodies (MVBs) ***(Lloyd TE, Atkinson R, Wu MN, Zhou Y, Pennetta G, Bellen HJ.*** *Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in Drosophila. Cell. 2002 Jan 25;108(2):261-9.).* Overexpression of Hrs in several systems blocked degradation of EGFR by trapping them in the early endosomes ***(Chin LS, Raynor MC, Wei X, Chen HQ, Li L.*** *Hrs interacts with sorting nexin 1 and regulates degradation of epidermal growth factor receptor. J Biol Chem. 2001 Mar 9;276(10):7069-78.).* Another molecule, tumor susceptibility gene 101 (TSG101) contains an Ubc enzyme-like domain that can recogonize ubiquitin ***(Ponting CP, Cai YD, Bork P.*** *The breast cancer gene product TSG101: a regulator of ubiquitination? J Mol Med. 1997 Jul;75(7):467-9.).* It complexes with hVPS28 and localized to endosomes that enclose internalized EGFR. Deletion of TSG 101 retards EGFR degradation and causes accumulation of ubiquitinated proteins on endosomes ***(Bishop N, Horman A, Woodman P.*** *Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates.J Cell Biol. 2002 Apr 1;157(1):91-101.).* In this study, when ubiquitination of EGFR was dramatically blocked in both system which is consistent with retarded receptor degradation, tracking the endocytosis of EGFR by fluorescence staining and colocalization with endosomal markers showed that majority of EGFR showed a delayed traffic from early endosome to late endosome, imply the ubiquitination as a crucial sorting signal that starts at very early step of endocytosis.

Cbl is a family that has three related mammlian gene products: Cbl, Cbl-b and Cbl-c (*Hamid, review*). Except for its evolutionarily conserved N-terminal region that is sufficient for the ubiquitination and degradation of EGFR (22),

Cbl and Cbl-b has an C-terminus which contains a SH3 domain-binding proline rich region (31) and several tyrosines which can be phosphorylated upon EGFR activation and interact with SH2-domain-containing proteins (32). One of the proteins is the SH3-SH2-SH3 adaptor protein Grb-2, which constitutively associated with Cbl by its N-terminal SH3 domain (33,34,35) and indirectly recruits Cbl by its SH2 domain to activated EGFR (36). It is still not understood that how the C-terminus of Cbl functions in the EGFR signaling pathway, as the N-terminus of Cbl is enough to enhance the ubiquitination of EGFR. Recent study found that C-terminus of Cbl may play a role in the ubiquitination of EGFR through its interaction with Grb-2. As overexpression of Grb2 causes Cbl dependent ubiquitination of EGFR Y1045F mutant that abolishes direct association of Cbl TKB domain with the receptors (*Yarden*). In our study, an EGFR Y1068F mutant which call off the major interaction between Grb-2 and EGFR greatly enhances ubiquitination and degradation of EGFR (Lei Duan, unpublished data), implying the Grb2-Cbl interaction limits the Cbl ubiquitination activity towards EGFR. Further study will be carried out to eventually elucidate the role of interaction of c-terminus of Cbl with Grb2 in the regulation of EGFR.

In conclusion, Cbl is the major ubiquitin ligase for ligand intiated ubiquitination of EGFR. Ubiquitination serves as a endosomal sorting signal for EGFR to be delivered from early endosome to lysosome for degradation whereas is dispensable for the internalization of EGFR.

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Figure Legends:

Fig 1: Ubiquitination and degradation of EGFR in Cbl $^{+/+}$ and Cbl $^{-/-}$ MEF cells. A, Characterize Cbl $^{+/+}$ and Cbl $^{-/-}$ MEF cells. Total cell lysate from both cell lines were blotted with anti-Cbl antibody to detect Cbl protein in those cells. B. Impaired ubiquitination of EGFR in Cbl $^{-/-}$ MEF cells. Both Cbl $^{+/+}$ (lane 1-4) and Cbl $^{-/-}$ MEF (lane 5-8) cells were stimulated with EGF after starvation, cells were harvested at the indicated time points (minutes), total cell lysate were IP with anti-EGFR antibody (528) and probed for ubiquitin (upper panel). The same membrane were striped and reprobed with anti-EGFR (middle panel) and anti-pY separately. C, Delayed degradation of EGFR in Cbl $^{-/-}$ MEF cells. Both Cbl $^{+/+}$ (lane 1-4)and Cbl $^{-/-}$ MEF (lane 5-8) cells were stimulated with EGF and harvested at the indicated time points (minutes), total cell lysate were blotted with anti-EGFR antibody (SC-03) to detect the degradation of EGFR, the changed protein level were density-measured as fold difference from unstimulated cells and labeled at the bottom.

Fig 2: Ubiquitination and degradation of EGFR in HA-Cbl reconstituted Cbl –/– MEF cells. A, Cbl –/– MEF cells were reconstituted with HA-Cbl by retroviral infection, both Cbl $^{+/+}$ and Cbl –/– MEF cells were infected with retroviral vector alone to be used as control. Total cell lysate from those cells were blotted with anti-Cbl antibody (SC-15) to detect either endogenous or reconstituted Cbl. The protein level of reconstituted Cbl is compared to the endogenous Cbl in Cbl $^{+/+}$ cells, marked at the bottom as fold difference of density. B. Ubiquitination of EGFR is restored in Cbl –/– MEF cells reconstituted with HA-Cbl. Cbl –/– MEF cells that either reconstitutred with HA-Cbl (lane 1-3) or vector (lane 4-6) were stimulated with EGF and harvested at the indicated time points. Total cell lysates were IP with anti-EGFR (528), then probe with anti-ubiquitin (upper panel). The same membrane was reprobed with anti-EGFR (lower panel). C, Degradation of EGFR is accelarated in Cbl –/– MEF cells reconstituted with HA-Cbl. The same cells as in B were stimulated with EGF and harvested at the indicated time points. Total cell lysates were blotted with anti-EGFR antibody to analyze the degradation of EGFR. The decreased EGFR level after stimulation were indicated as fold difference of density from that in the non-stimulated cells at the bottom.

Fig 3 : Internalization and downregulation of EGFR in Cbl $^{+/+}$ and Cbl –/– MEF cells. A. Left panel: Cbl $^{+/+}$ and Cbl –/– MEF cells were surface bound with Alexa-fluo488-conjugated EGF and allowed to internalize EGF for a time course as described in method. The percentage internalization of EGF were calculated from the mean fluorescence intensity of total cell surface EGF versus that of internalized EGF at the indicated time points. Right panel: Cbl –/– MEF cells were transfected with HA-Cbl or with vector and internalization were done as described above. B. Internalization of EGFR were shown by fluorescence staining of EGFR before and after (5 min) EGF stimulation in both Cbl $^{+/+}$ (left panel) and Cbl –/– (right panel) cells. C. Left panel: Cbl $^{+/+}$ and Cbl –/– MEF cells were stimulated with EGF (25ng/ml) and harvested at the indicated time points. Cells were alive stained with anti-EGFR (528) for the cell surface pool of EGFR. Percentage downregulation were calculated from the mean fluorescence intensity of total cell surface EGFR without stimulation versus remained cell surface EGFR after stimulation. Right panel: Cbl –/– MEF cells were transfected with HA-Cbl or with vector, Cbl $^{+/+}$ cells were transfected with vector. Downregulation of EGFR were done as described above.

Fig 4: Ubiquitination and degradation of EGFR in E36 and Ts20 cells. Ts 20 Cells (Panel A) or E36 cells (Panel B) stably transfected with human EGF receptor were cultured at either 30°C (Lane 1-3) or 42°C (lane 4-6) for 4 hours, then stimulated with EGF. Anti-EGFR IPs from cell lysates prepared at the indicated time points were blotted with an anti-ubiquitin antibody followed by anti-EGFR reprobing. Ts20 cells in panel C and E36 cells in panel D: cell lysates from the same experiment were blotted with an anti-EGFR antibody to detect the degradation of EGFR at the indicated time points. The degradation of EGFR after stimulation were indicated as fold difference of density from that in the non-stimulated condition.

Fig 5: Internalization of EGFR in E36 and Ts20 cells. E36 (left panel) or TS20 cells (right panel) were cultured at the permissive or non-permissive temperatures, as indicated. Cells were allowed to bind the Alexa-fluo488-conjugated EGF at 4°C for 30 minutes,and returned to 37°C to allow internalization. At the indicated time points, the cells were acid washed (pH2.8) to remove the non-internalized labeled EGF, the internalized EGF signals were measured using flow cytometry. Mean fluorescence intensity of cells without an acid wash (total surface pool) was used to calculate the percentage of internalized EGF.

Fig 6: Colocalization of EGFR with transferrin in E36 and Ts20 cells. Cells were cultured at the permissive or non-permissive temperature and stimulated with EGF, as indicated. The cells were loaded with Alexa-fluo488-conjugated transferrin for 5 minutes before harvesting to visualize the early endosome/recycling compartment. The cells were fixed and stained with an anti-EGFR antibody (mAb 528) followed by Cy3-conjugated anti-mouse antibody, and analyzed by confocal microscopy to visualize the colocalization of EGFR with transferrin. Under permissive temperature, both E36 (6A) and Ts20 (6B) cell lines showed the internalized EGFR in peripherally-distributed (6A:b1; 6B:b1) endosomes at 5 min, which clustered in the center of the cell at 30 min (6A:c1; 6B: c1). At 5 min, EGFR colocalized with transferrin (Yellow, 6A: b3, b4; 6B: b3, b4), but the colocalization was lost at 30 min (6A: c3, c4; 6B: c3, C4).

Under the non-permissive temperature condition, the vesicle distribution (6A: e1 f1) and co-localization of EGFR with transferrin (6A: e3, e4, f3, f4) in E36 cells was indistinguishable from that at the permissive teperature In contrast, EGFR remained peripherally localized (6B: f1) and continued to colocalize with transferrin (yellow, 6B: f3, f4) at 30 min in TS20 cells grown at the non-permissive temperature.

Fig 7: Colocalization of EGFR with LAMP-1 in E36 and Ts20 cells. Cells were processed as in Fig.6 and stained with mAb anti-EGFR (visualized with Cy3-conjugated anti-mouse) and rabbit anti-LAMP1 (late endosome marker; visualized with Cy2-conjugated goat anti-Rabbit) antibodies, and analyzed by confocal microscopy. At the permissive temperature, colocalization of EGFR and LAMP1 was observed at 30 min after EGF stimulation in both E36 cells (yellow, b3, b4) and TS20 (e3, e4) cells. At the non-permissive temperature, E36 cells continued to show the same pattern (c3, c4). In contrast, the EGFR remained peripherally-distributed (f1) and showed reduced colocalization with LAMP1 (f4) in TS20 cells.

Fig 1

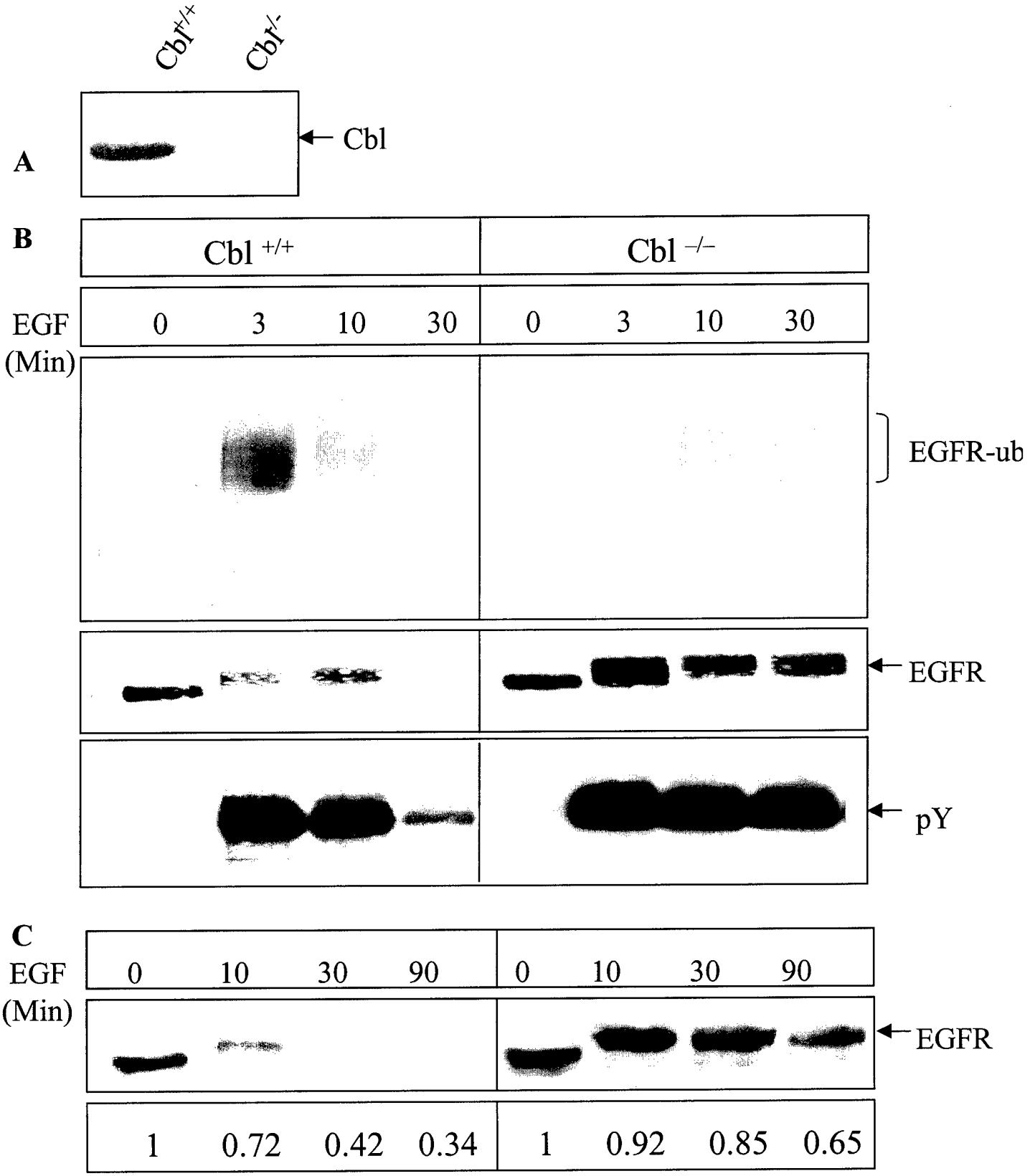

Cbl+/+
Cbl-/-
A
Cbl
B
Cbl +/+
Cbl −/−
EGF
(Min)
0
3
10
30
0
3
10
30
EGFR-ub
EGFR
pY
C
EGF
(Min)
0
10
30
90
0
10
30
90
EGFR
1
0.72
0.42
0.34
1
0.92
0.85
0.65

Fig 2

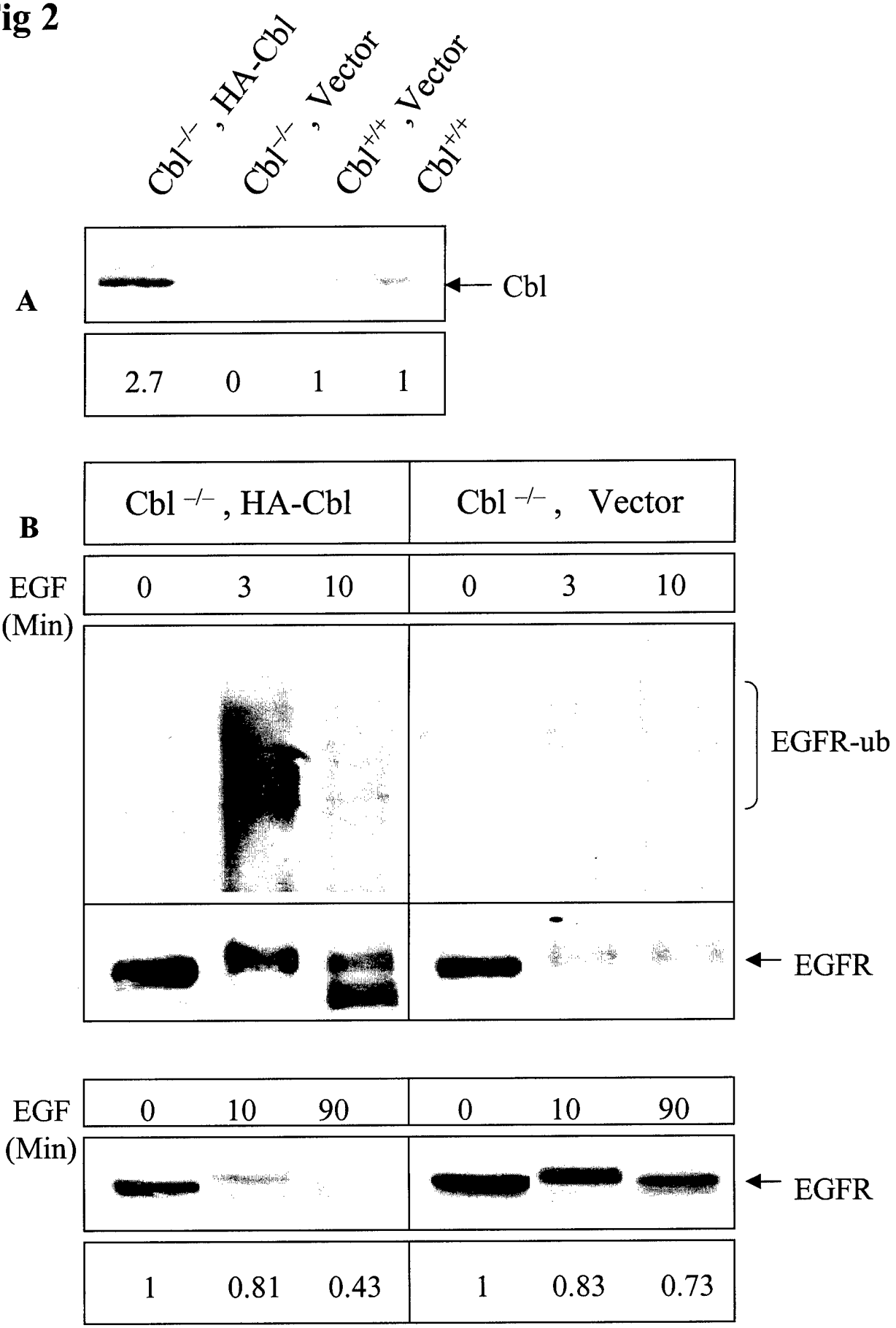

Cbl−/− , HA-Cbl
Cbl−/− , Vector
Cbl+/+ , Vector
Cbl+/+
A
Cbl
2.7
0
1
1
B
Cbl −/− , HA-Cbl
Cbl −/− , Vector
EGF (Min)
0
3
10
0
3
10
EGFR-ub
EGFR
C
EGF (Min)
0
10
90
0
10
90
EGFR
1
0.81
0.43
1
0.83
0.73

Fig 3

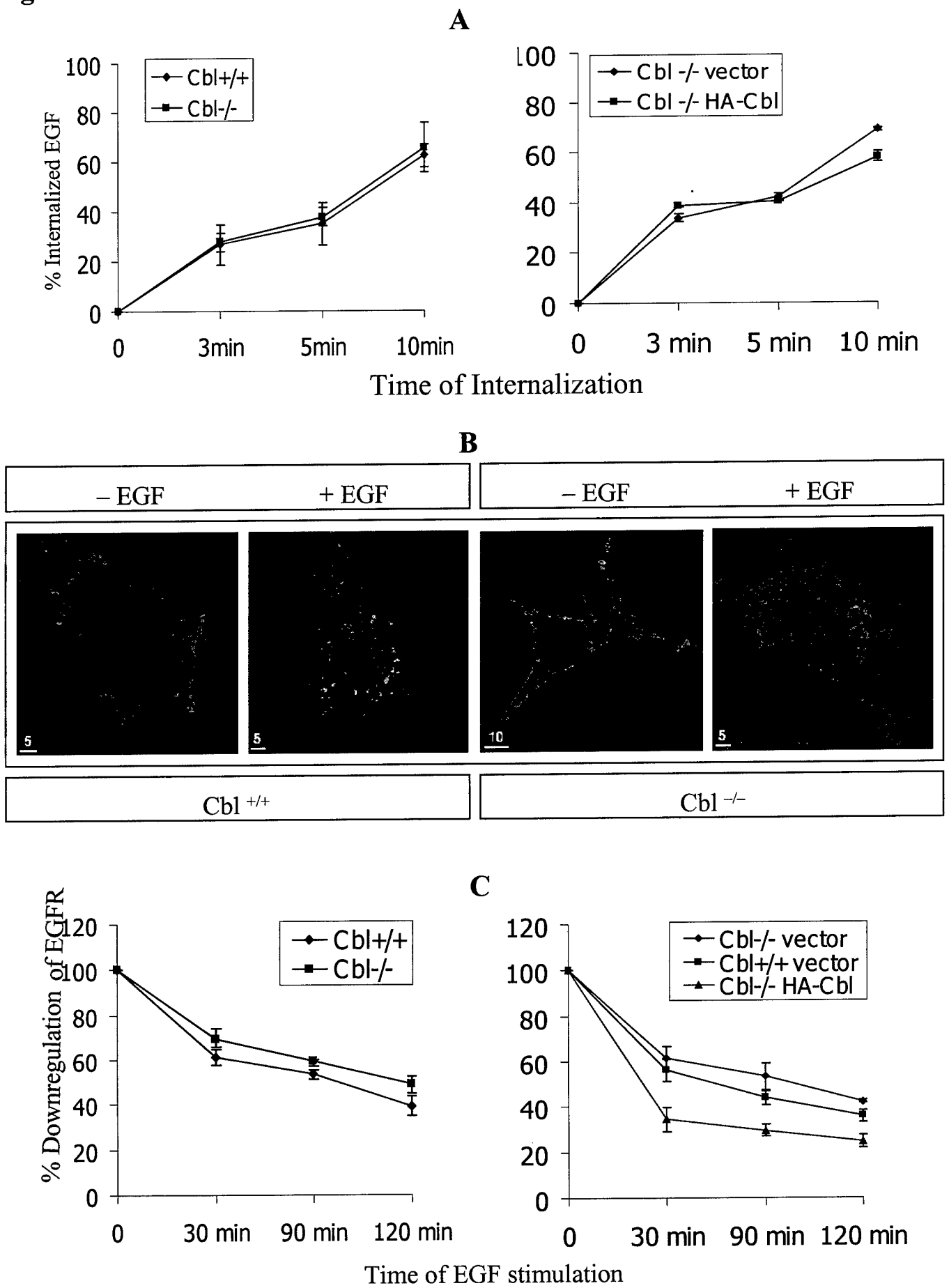

A
% Internalized EGF
Cbl+/+
Cbl-/-
Cbl -/- vector
Cbl -/- HA-Cbl
0
3min
5min
10min
3 min
5 min
10 min
Time of Internalization
B
– EGF
+ EGF
– EGF
+ EGF
Cbl +/+
Cbl −/−
C
% Downregulation of EGFR
Cbl+/+
Cbl-/-
Cbl-/- vector
Cbl+/+ vector
Cbl-/- HA-Cbl
0
30 min
90 min
120 min
Time of EGF stimulation

Fig 4

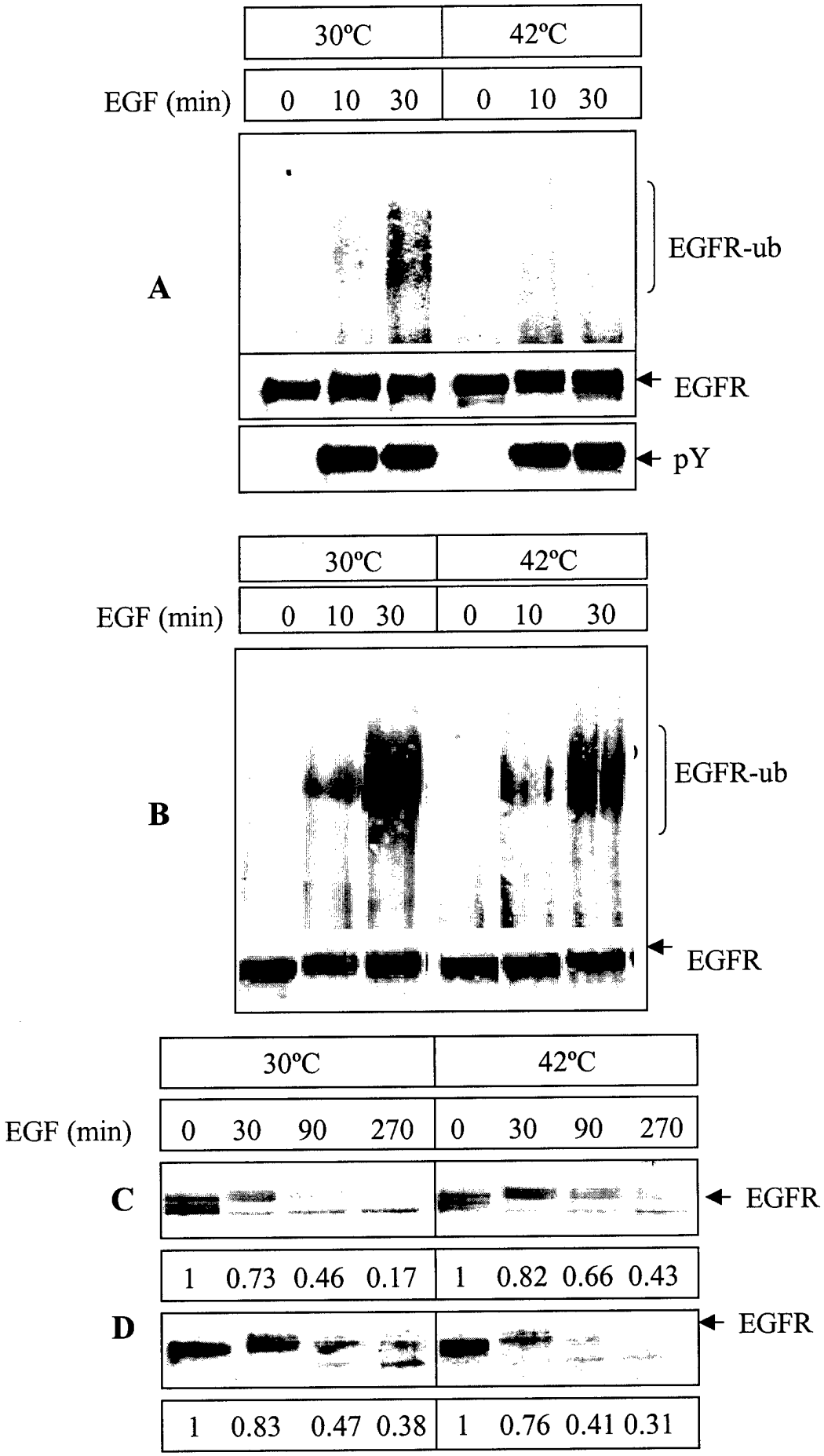

30°C
42°C
EGF (min)
0 10 30 0 10 30
A
EGFR-ub
EGFR
pY
30°C
42°C
EGF (min)
0 10 30 0 10 30
B
EGFR-ub
EGFR
30°C
42°C
EGF (min)
0 30 90 270 0 30 90 270
C
EGFR
1 0.73 0.46 0.17 1 0.82 0.66 0.43
D
EGFR
1 0.83 0.47 0.38 1 0.76 0.41 0.31

Fig 5

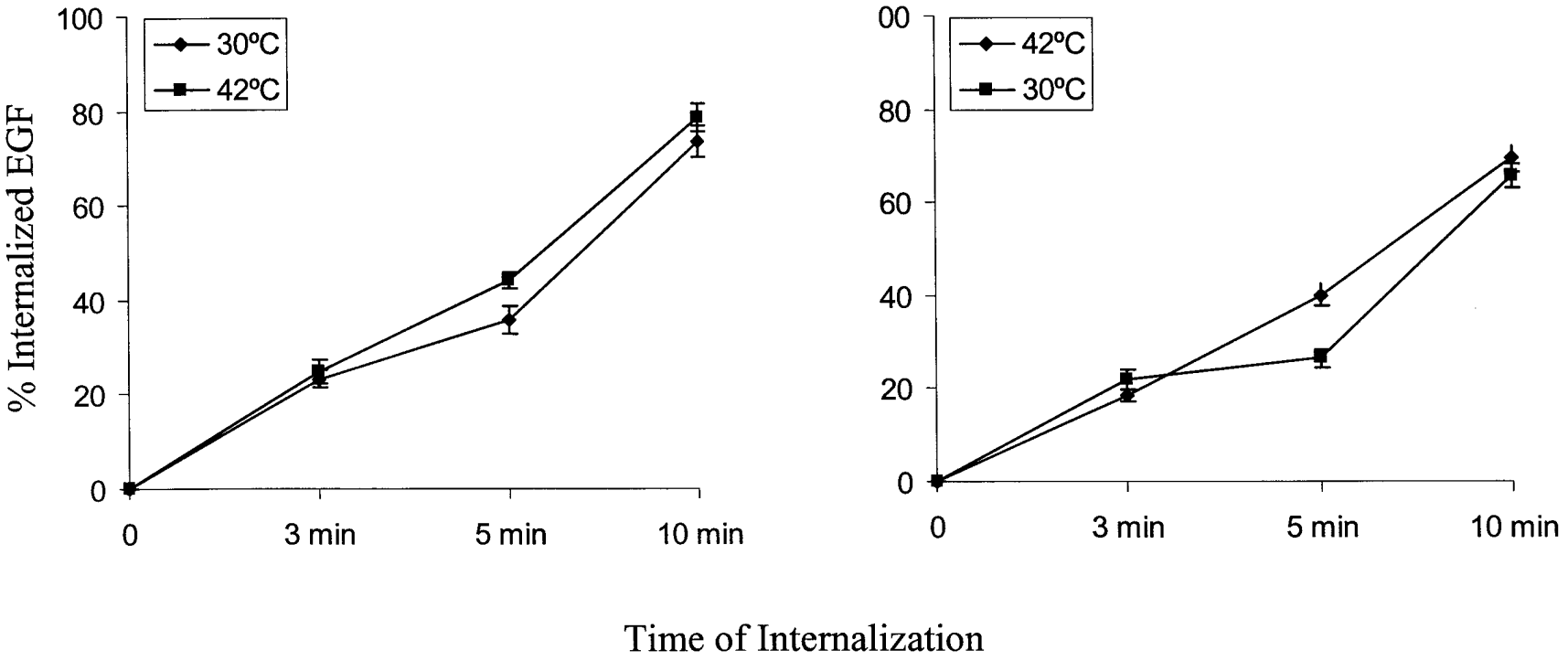

% Internalized EGF
100
80
60
40
20
0
30°C
42°C
0
3 min
5 min
10 min
00
80
60
40
20
0
42°C
30°C
0
3 min
5 min
10 min
Time of Internalization

Fig 6A

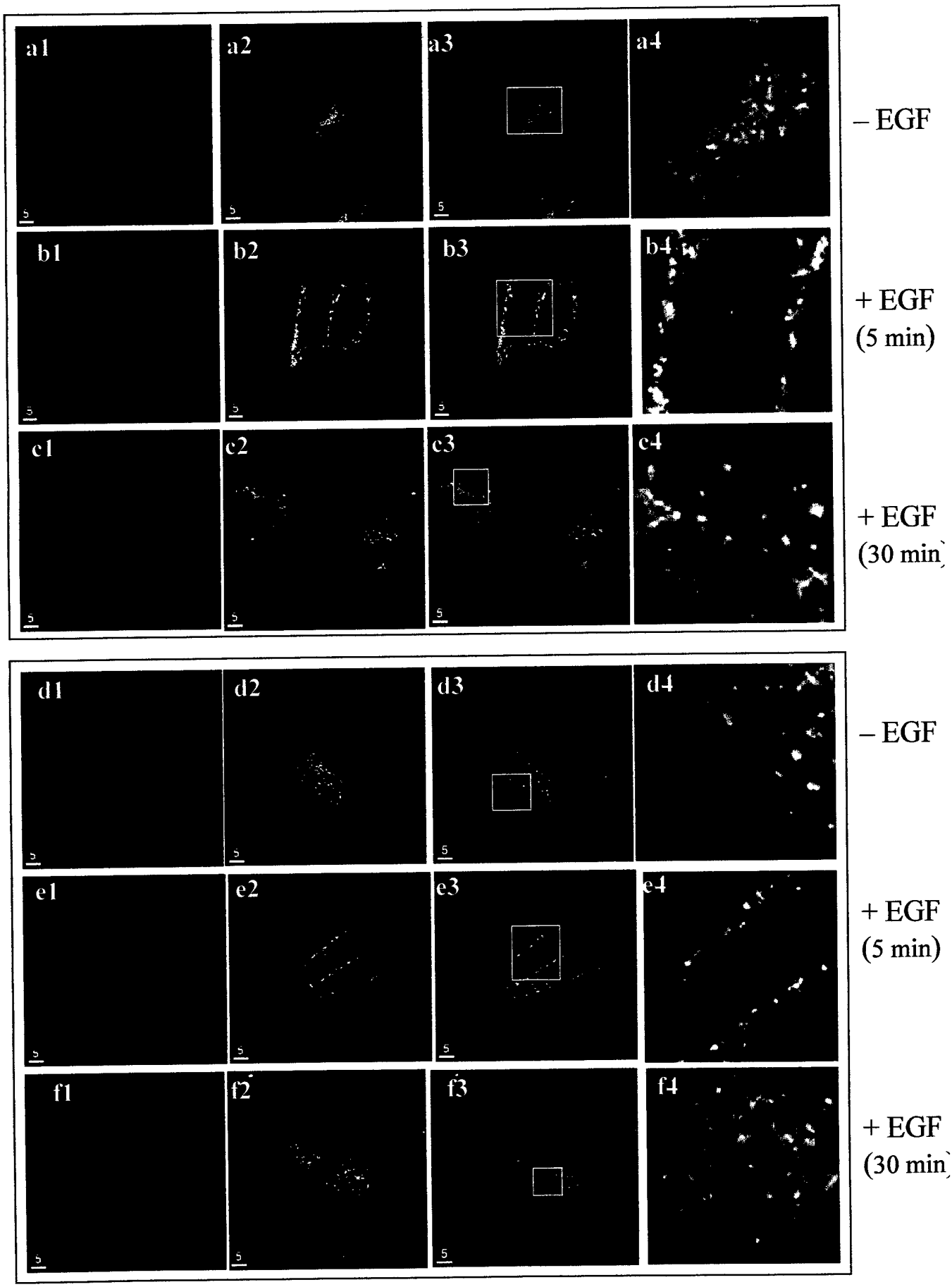
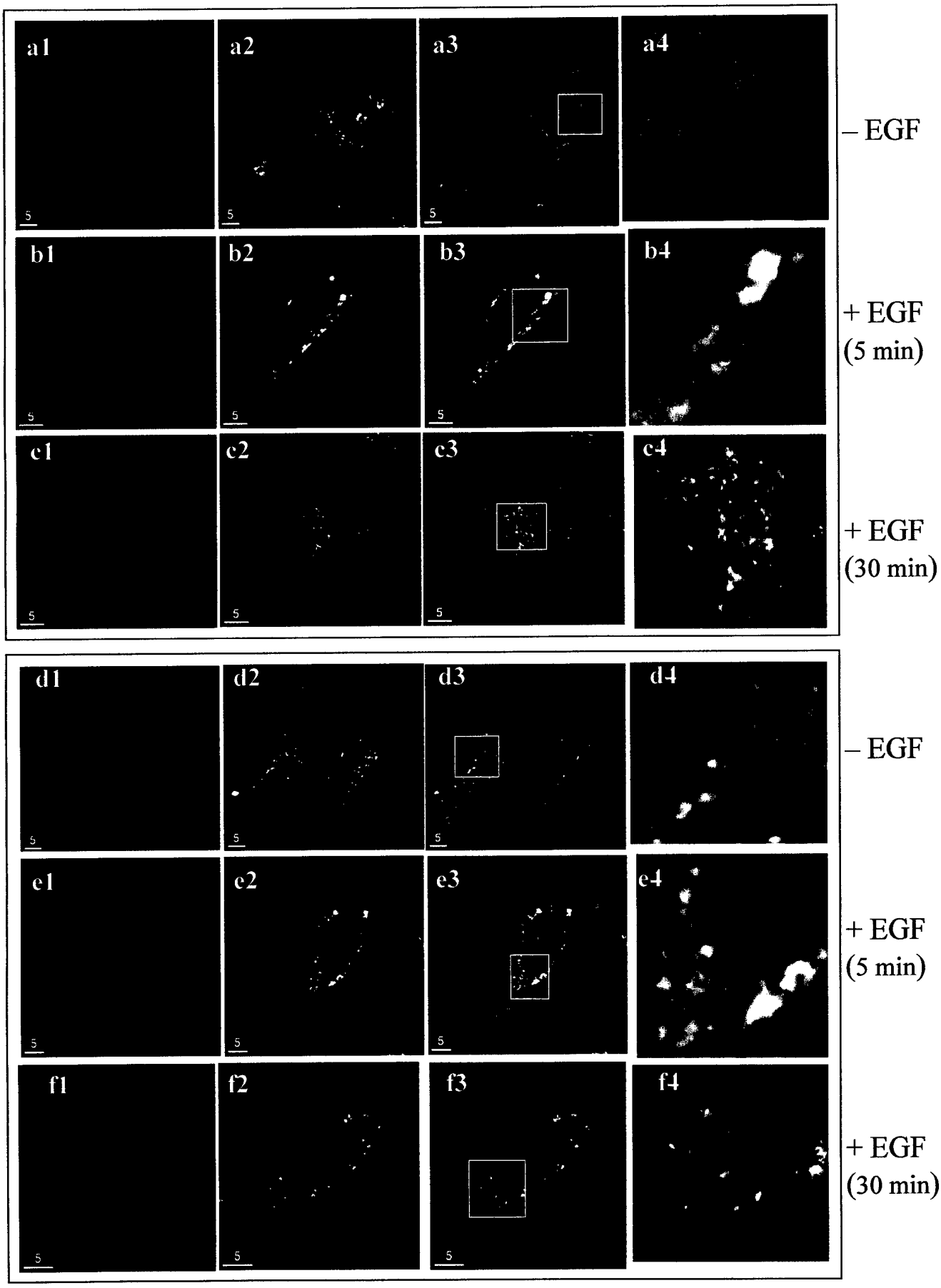

a1
a2
a3
a4
– EGF
b1
b2
b3
b4
+ EGF (5 min)
c1
c2
c3
c4
+ EGF (30 min)
d1
d2
d3
d4
– EGF
e1
e2
e3
e4
+ EGF (5 min)
f1
f2
f3
f4
+ EGF (30 min)
EGFR
Transferrin
Merge

Fig 6B


a1
a2
a3
a4
– EGF
b1
b2
b3
b4
+ EGF (5 min)
c1
c2
c3
c4
+ EGF (30 min)
d1
d2
d3
d4
– EGF
e1
e2
e3
e4
+ EGF (5 min)
f1
f2
f3
f4
+ EGF (30 min)
EGFR
Transferrin
Merge